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Doctorado en Ciencias en Conservación y Aprovechamiento de Recursos Naturales

“Fitoquímica y actividad biológica de *Prosthechea karwisnkii* (Orchidaceae)”

T E S I S

QUE PARA OBTENER EL GRADO ACADÉMICO DE

Doctor en Ciencias en Conservación y Aprovechamiento de Recursos Naturales

P R E S E N T A:

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Dra. Luicita Lagunez Rivera
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INSTITUTO POLITÉCNICO NACIONAL
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ACTA DE REVISIÓN DE TESIS

En la Ciudad de Oaxaca Siendo las 11:00 horas del día 22 del mes de octubre del 2018 se reunieron los miembros de la Comisión Revisora de la Tesis, designada por el Colegio de Profesores de Estudios de Posgrado e Investigación de CIIDIR UNIDAD OAXACA para examinar la tesis titulada:

Fitoquímica y actividad biológica de *Prosthechea karwisnkii* (Orchidaceae)

Presentada por el alumno:

Rojas	Olivos
Apellido paterno	Apellido materno

Nombre(s) Alejandra

Con registro: | A | 1 | 1 | 0 | 2 | 5 | 4 |

aspirante de:

Doctorado en Ciencias en Conservación y Aprovechamiento de Recursos Naturales

Después de intercambiar opiniones los miembros de la Comisión manifestaron ***APROBAR LA TESIS***, en virtud de que satisface los requisitos señalados por las disposiciones reglamentarias vigentes.

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CENTRO INTERDISCIPLINARIO DE INVESTIGACIÓN PARA EL DESARROLLO INTEGRAL REGIONAL C.I.I.D.I.R. UNIDAD OAXACA I.P.N.

CONTENIDO

Dedicatoria

A mi esposo *Lezghi Abdiel*, porque nada de mi vida estaría completo sino lo comparto contigo.

A mis hijos *Pedro Andreu* y *Santiago Nicolás*:
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Resumen

Prosthechea karwinskii es una orquídea epífita, endémica de México, con uso cultural y en la medicina tradicional. En este trabajo se obtuvieron extractos hidroetanólicos e hidrometanólicos de los pseudobulbos, hojas y flores de esta especie, aplicando previamente sonicación, a los cuales se les evaluó su capacidad antioxidante. Posteriormente, se evaluó la actividad de los tres extractos hidroetanólicos en ratas Wistar con síndrome metabólico inducido, evaluando su efecto en tejido adiposo y en los niveles de colesterol, glucosa y triglicéridos. Considerando los resultados obtenidos en la evaluación biológica de cada parte de la planta, el mucílago obtenido del pseudobulbo fue evaluado por su posible efecto protector del riñón relacionado con el aumento del nivel de adiponectina y la disminución del tejido adiposo; se realizó también la caracterización del mucílago mediante FT-IR. Los resultados demostraron que el extracto de hojas presentó un mayor índice de actividad antioxidante y contenido de flavonoides totales, seguido por los extractos de flores y pseudobulbos. Con respecto al efecto de la especie en síndrome metabólico, el extracto de pseudobulbo tuvo una mayor disminución del tejido adiposo; el de hojas mayor efecto reductor en el nivel de glucosa y el de flores mayor reducción en los niveles de colesterol y triglicéridos. El extracto tuvo también un efecto protector para el riñón, favoreciendo un aumento de adiponectina y disminución de ácido úrico, creatinina y urea. El análisis por FTIR del mucílago y polisacárido aislado del pseudobulbo de *Prosthechea karwinskii* evidenció la presencia de grupos funcionales similares a los del glucomanan, por lo que podría tener propiedades similares a las reportadas en polisacáridos de la familia de manoglicanos.

PALABRAS CLAVE: etnofarmacología, plantas medicinales, síndrome metabólico, actividad antioxidante, manoglicanos.

Abstract

Prosthechea karwinskii is an epiphytic orchid, endemic to Mexico, with cultural use since prehispanic times and used in traditional medicine in some regions of Oaxaca to control diabetes and other conditions. In this work, hydroethanolic and hydrometanolic extracts of the pseudobulbs, leaves and flowers of this plant were obtained, previously applying sonication, to which their antioxidant capacity was evaluated. Subsequently, the activity of the three hydroethanolic extracts in Wistar rats with induced metabolic syndrome was evaluated, assessing their effect on adipose tissue and on cholesterol, glucose and triglyceride levels. Considering the results obtained in the biological evaluation of each part of the plant, the mucilage obtained from the pseudobulb was evaluated to evaluate its protective effect of the kidney related to the increase of the adiponectin level and the decrease of the adipose tissue, the characterization of the mucilage using FT-IR. The results showed that the leaf extract showed a higher index of antioxidant activity and content of total flavonoids, followed by flowers and pseudobulbs. With respect to the effect of the species on the metabolic syndrome, the pseudobulb extract showed a greater decrease in adipose tissue, the leaves extract had greater reducing effect in the glucose level, and the flowers extract of flowers greater reduction in the cholesterol and triglycerides levels. In addition, the protective effect of the kidney was demonstrated by promoting an increasing in adiponectin levels and a decreasing of in the uric acid, creatinine and urea levels. The analysis by FTIR of the mucilage and the polysaccharide isolated from the pseudobulb showed the presence of functional groups similar to those found in glucomannan, considering a possible similarity in the properties reported for polysaccharides of the mannoglycan family.

Keywords: ethnopharmacology, medicinal plants, metabolic syndrome, antioxidant activity, manoglicans.

I. Introducción

El uso de plantas, extractos o derivados químicos obtenidos de ellas para el tratamiento de enfermedades, es una modalidad terapéutica que ha resistido el paso del tiempo. La medicina tradicional ha sido la base para el desarrollo de los fármacos sintéticos que se utilizan actualmente. Sin embargo, aún existen comunidades indígenas o campesinas dónde el uso de las plantas se ha conservado como una práctica común para la atención de los problemas de salud, siendo importante corroborar científicamente el uso medicinal de especies vegetales considerando los métodos de preparación tradicionales usados por las comunidades así como posibles usos alternativos.

I.1 Orquídeas medicinales

Según Hossain (2011) se conocen casi 2,000 especies de orquídeas que son utilizadas en la medicina tradicional alrededor del mundo. Muchas de ellas han sido estudiadas por la importancia de los metabolitos secundarios responsables de su actividad biológica. Entre estos compuestos se encuentran alcaloides y flavonoides, fenantrenos, bibenziles, fluorenonas y sesquiterpenos (Liu et al., 2004; Hossain, 2011). En México se han reportado más de 10 géneros de orquídeas con uso medicinal y festivo, entre los que destacan especies del género *Arpophyllum, Barkeria, Bletia, Catasetum, Cyrtopodium, Epidendrum, Laelia, Myrmecophyla, Oncidium, Prosthechea, Rhyncosthele;* reportadas con uso medicinal para el tratamiento de heridas, disentería, diabetes, tos, prevención de abortos y cáncer (Hágsater et al.,2005).

Prosthechea karwinskii (Mart.) J.M.H. Shaw (Fig. 1), es una orquídea endémica de México, se distribuye en los estados de Oaxaca, Guerrero, Morelos, Michoacán, Puebla y Veracruz (Fig. 2) (Avendaño-Vásquez y Cruz-Lustre, 2007). Crece como epífita en bosques de encino o pino-encino, raramente en matorrales xerófilos, entre los 2,000 y 2,400 m de elevación. En Oaxaca crece en la parte interna de las montañas que rodean los Valles Centrales, en encinares estacionalmente secos de *Quercus acutifolia, Q. laeta*, *Q. liebmannii, Q. magnoliifolia, Q. obtusata, Q. peduncularis, Q. rugosa y Q. urbanii*, los cuales utilizan como árboles hospederos (Avendaño-Vásquez y Cruz-Lustre, 2007). En Oaxaca esta especie se conoce con los nombres de 'monja amarilla', 'flor de mayo', 'flor castigada' e 'itan chaca cúan', 'ita ndeka amarilla' (Cruz-García et al., 2014).

En algunas regiones de Oaxaca y Michoacán *Prosthechea karwinskii* es utilizada en la medicina tradicional para el tratamiento de heridas y quemaduras, problemas de las vías respiratorias como tos e infecciones de la garganta, facilitar la labor de parto y para el control de la diabetes (Cruz-García et al., 2014). Por otro lado, esta especie es muy apreciada en Oaxaca por su valor ornamental y ceremonial; durante la celebración de la Semana Santa, que coincide con el periodo de floración de la planta, en varias comunidades del estado se elaboran adornos efímeros con las flores que son colocados en Iglesias, altares, así como en casas. Es una de las orquídeas más comercializada en mercados locales, donde los ejemplares son ofrecidos como adorno efímero durante el período de floración. Además es también una especie muy apreciada en el cultivo rústico en muchas comunidades donde crece la planta (Fig. 3–4) (Hágsater et al., 2005; Solano et al., 2010; Cruz-García et al., 2014).

I.2 Mucílago de orquídeas

En el Códice Florentino (Sahagún, 1829) y en el Jardín Botánico (Navarro, 1992) se describe el arte plumario realizado por los *amantecas* (artesanos) aztecas (Fig. 5). En este arte fueron importantes las orquídeas, pues de sus pseudobulbos o cormos se obtenía el adhesivo empleado para pegar las plumas. El termino *tzacuhtli* se utilizó para nombrar a las orquídeas empleadas para este fin o bien, para el mucílago que se obtenía de ellas, pues en Náhuatl tzacuhtli significa sustancia gomosa, engrudo o mucílago. Entre las orquídeas usadas en la plumaria se encuentra *Prosthechea citrina* (Urbina, 1903), pero es probable que en realidad se trate de su especie hermana (o que también fuera utilizada), *P. karwinskii*, con la cual ha sido confundida en el pasado debido a la gran similitud entre ellas (Cruz-García et al., 2014). Para obtener el tzacuhtli el amanteca ponía a deshidratar los seudobulbos de la orquídea y luego los hacía polvo; cuando se requería el mucílago, ese polvo era humedecido con agua. Los tzacuhtli también se emplearon durante la época de la Colonia como adhesivo para amasar una pasta de caña de maíz con la cual se elaboraban los “Cristos de caña” y otras imágenes religiosas que, por su ligereza, eran usados en las procesiones religiosas (Hágsater et al., 2005).

El mucílago obtenido de algunas especies de orquídeas del género *Cyrtopodium* y *Dendrobium* entre otras, han sido evaluadas debido a sus propiedades para modificar la textura de productos alimenticios. El polisacárido más reportado en este aspecto es el Salep, usado en países mediterráneos como aromatizante, saborizante, estabilizante y aglutinante de helados y bebidas, es obtenido a partir de pseudobulbos de orquídeas terrestres y es considerado una fuente importante de glucomanan, cuya estructura química está formada principalmente por dos monómeros lineales β-D-manosa y α-D-glucosa. Las propiedades reológicas del salep como ingrediente único y su combinación con otros agentes espesantes como la goma guar, xhantana y alginato, han comprobado sus efectos en la calidad final de productos alimenticios (Kayacier & Dogan, 2006; Pourjavadi et al., 2012.).

I.3 Síndrome metabólico

Actualmente el estilo de vida sedentaria y la calidad de la alimentación han sido factores determinantes para el desarrollo de enfermedades degenerativas cuyos síntomas se manifiestan lentamente. Tal es el caso de los padecimientos que en conjunto constituyen la patogénesis del síndrome metabólico. A pesar de existir tratamientos con fármacos facilitados por los servicios de salud pública, se ha recomendado incluir en el tratamiento del síndrome metabólico el uso de plantas medicinales o sustituirlo por éstas para contrarrestar los efectos secundarios de los fármacos o bien, ser un coadyuvante contra los padecimientos. Además, el empleo de la fitoterapia es un recurso con una mayor disponibilidad económica para toda la población y, cabe recordar que las plantas medicinales han sido en esencia la base de la elaboración de fármacos sintéticos (Cai et al., 2001; Gurrola-Díaz et al.,2010). Una de las áreas importantes en la que compuestos de origen vegetal han contribuido con éxito, es la investigación en enfermedades cardiovasculares. En este caso se hace hincapié en la hipertensión arterial, la cual ha sido denominada como la plaga silenciosa del siglo XXI. Sin embargo existen mayores casos de síndrome metabólico en México que en poblaciones caucásicas como lo menciona Lerman-Garber et al. (2004) quién también indica la definición considerada por la Organización Mundial de la Salud para determinar si una persona presenta Síndrome metabólico basado en las siguientes características:

“El paciente debe presentar diabetes o concentración de glucosa en ayunas anormal o bien resistencia a la insulina determinada por el método de homeóstasis y al menos dos de los siguientes criterios”:

1.-Relación cintura/cadera >0.9 en hombres y >0.85 en mujeres

2.-Triglicéridos ≥ 150 mg/dl

3.-Colesterol HDL < 35 mg/dl en hombres y < 39 mg/dl en mujeres

4.-Tasa de excreción de albúmina en orina >20 µg/min

5.-Presión arterial ≥ 140/90 mmHg

De acuerdo con Gogia y Agarwal, 2004 (citados por Merino-Aguilar, 2014), el síndrome metabólico presenta los siguientes tres puntos cruciales en su patogénesis:

1.- Obesidad y alteración en el tejido adiposo: factor principal para desarrollar síndrome metabólico que también contribuye a desarrollar hipertensión, altos niveles de colesterol y triacilglicéridos en la sangre y bajas concentraciones de lipoproteínas de alta densidad (HDL), que están asociadas a enfermedades cardiovasculares.

2.-Resistencia a la insulina relacionada con disfunción en los adipocitos.

3.- Factores moleculares independientes involucrados en las respuestas inmunológica, vascular y hepática.

Se estima que el síndrome metabólico se presenta en el 62% de los sujetos que padecen diabetes, el 34% de los que son hipertensos, 37% de quienes son hipertrigliceridémicos, el 20% de quienes presentan bajos niveles de colesterol HDL y el 42% de las personas que tienen micro-albuminuria (Aguilar-Salinas et al., 2005). Conforme es mayor el número de problemas asociados al síndrome metabólico, tiende a ser más significativa la resistencia a la insulina y mayor el riesgo cardiovascular (Aguilar-Salinas et al., 2005).

I.4 Capacidad antioxidante de las orquídeas

Se ha reconocido el efecto de los compuestos fenólicos para proteger a las células de los daños causados por estrés oxídativo, debido a la capacidad antioxidante de estos compuestos presentes en diversas plantas, ayudando a proteger contra enfermedades cardiovasculares, cancerígenas, inflamatorias y patologías (Kalita et al., 2013; Lavate et al., 2013; Stanojević et al., 2009;Percival, 1998;). Entre las diversas especies con potencial antioxidante se encuentran los reportes para algunas orquídeas sobre su efecto citotóxico debido a compuestos fenólicos, como los presentes en *Laelia anceps* Lindl. (Jimarez-Montiel, 2009), cuya actividad ha sido evaluada en líneas celulares de cáncer de mama. Otra especie de orquídea reportada con elevada capacidad antioxidante ha sido *Prosthechea michuacana* (Lex.) W.E. Higgins (Neira-González, 2009), de la cual se identificaron compuestos fenólicos en los extractos de raíz y pseudobulbo. Hernández-Galicia (2010) reportó la actividad antiinflamatoria y citotóxica en líneas celulares de próstata, leucemia, colon y mama, del extracto de las hojas de *Oncidium sphacelatum* Lindl., adjudicando el efecto a las flavonas identificadas en ese extracto.

Para la especie *Dendrobium speciosum* Sm. se determinó la capacidad antioxidante de sus extractos metanólicos de hojas y pseudobulbos usando el método DPPH, los resultados demostraron que el extracto de hojas fue más eficiente en la captación de radicales libres y tuvo un mayor porcentaje de flavonoides y polifenoles en comparación con el extracto de sus tallos (Moretti et al., 2013).

Finalmente, respecto a *Prosthechea karwinskii* sólo se había reportado la presencia de compuestos fenólicos en el extracto de toda la planta (Mijangos-Ricardez, 2010): hidroxitirosol, tirosol, apigenina-7-glucosido, ácido cafeíco, vainillina, acido *p*-cumárico y ácido ferúlico; todos ellos con reportes de capacidad antioxidante importante. A uno de estos compuestos, apigenina, se le reportan propiedades antioxidante, antimicrobiana, antiviral, antibacteriana, antiinflamatoria, cardioprotectora y anticancerígena. En otra especie del género, *Prosthechea varicosa* (Lindl.) W.E Higgins, también se han reportado compuestos fenólicos: apigenina, hidroxitirosol, tirosol, ácido ferúlico, ácido p-cumárico, vainillina, ácido cafeíco, oleuropeína, ácido o-cumárico y leuteolina-7-glucósido (Mijangos-Ricardez, 2010).

I.5 Objetivos

Objetivo general

Obtener los extractos de cada parte de la orquídea *Prosthechea karwinskii*, caracterizar su capacidad antioxidante y evaluar su efecto en los parámetros característicos del síndrome metabólico

La tesis se ha estructurado en cuatro capitulos el primero corresponde a la introducción y los restantes refieren las tres etapas del trabajo de tesis doctoral, cada una de ellas corresponde con los capítulos II al IV aquí presentados. A continuación se describe el objetivo partiular y una descripción de cada capítulo.

Capitulo II

Objetivo: Determinar la capacidad antioxidante de los extractos de *Prosthechea karwisnkii* obtenidos por sonicación.

Este capítulo ya ha sido publicado como artículo científico

Alejandra Rojas-Olivos, Rodolfo Solano-Gómez, Manuel Jiménez-Estrada, Blanca Yakelina Silva-Valeriano, Luicita Lagunez-Rivera. Antioxidant Capacity of *Prosthechea karwi*nskii extracts obtained by sonication. *Journal of Chemical Biological and Physical Sciences* 2014 (3), 4.

En este trabajo se obtuvieron extractos del pseudobulbo, hoja y flor de la especie a partir de diferentes métodos usando dos solventes diferentes como etanol:agua y metanol:agua, comparándo con aquellos extractos a los cuáles se les aplicó previamente sonicación para posteriormente determinar la capacidad antioxidante de cada extracto y evaluar su efecto *in vitro* en las diferentes líneas celulares de cáncer. Los resultados obtenidos demostraron que el tipo de solvente y el tratamiento previo de sonicación antes de la extracción influyen en el índice de actividad antioxidante (IAA), el extracto que demostró mayor IAA fueron los obtenidos a partir de las hojas usando como solvente combinación de etanol:agua y metanol:agua así como el tratamiento previo de sonicación, estos mismos extractos demostraron mayor efecto inhibitorio en líneas celulares de cáncer de mama.

Capitulo III

Objetivo: Realizar la evaluación biológica de los extractos hidroalcoholicos de la orquídea**.**

Este capítulo ya ha sido publicado como artículo científico:

Alejandra Rojas-Olivos,Rodolfo Solano-Gómez, Alfonso Alexander-Aguilera, Manuel Jiménez-Estrada, Stefan Zilli-Hernández, Luicita Lagunez-Rivera. Effect of *Prosthechea karwinskii* on obesity and dislipidemia in Wistar rats. *Alexandria Journal of Medicine.* 2017 (53); 311-15.

Para este segundo objetivo únicamente fueron evaluados los extractos hidroetanólicos (etanol:agua) obtenidos a partir de las hojas, flores y pseudobulbos, debido a que la especie no habia sido evaluada *in vivo* se realizó la evaluación biológica considerando el método de extracción tradicional por maceración (etanol:agua). Se utilizó el modelo biológico de rata Wistar, los animales fueron alimentados durante 3 meses con sacarosa con la finalidad de inducir el síndrome metabólico, al finalizar se les administró 200 mg/kg de extracto de cada parte de la especie vegetal (pseudobulbo, hojas y flores) durante 13 días posteriormente los animales fueron sacrificados para evaluar los parámetros séricos de colesterol, glucosa y triglicéridos, además de obtener el tejido adiposo abdominal, epididimal y pericárdico. Los resultados obtenidos demostraron que todos los extractos redujeron los parámetros séricos evaluados; sin embargo, el que tuvo mayor efecto reductor en glucosa fue el extracto de hojas, el de seudobulbos tuvo mayor efecto reductor en tejido adiposo, colesterol y triglicéridos.

Capitulo IV

Objetivo: Caracterizar el mucílago de la orquídea y evaluar su efecto protector del riñón en ratas con síndrome metabólico inducido:

Este capítulo ha sido sometido para su publicación como artículo científico:

Alejandra Rojas-Olivos, Rodolfo Solano-Gómez, Alfonso Alexander-Aguilera, Ida Soto-Rodríguez, Manuel Jiménez-Estrada, Luicita Lagunez-Rivera. Kidney protection and hypolipidemic effect of *Prosthechea karwinskii* (Orchidaceae) mucilage in Wistar rats (manuscrito en revisión).

Este ultimo objetivo fue enfocado únicamente a evaluar el mucílago obtenido a partir del extracto hidroetanólico del pseudobulbo, se siguió la misma metodología de inducción al síndrome metabólico del modelo murino planteada en el objetivo anterior, la concentración administrada del mucílago fue de 200 mg/kg. Además, en este trabajo se comparó el efecto del mucílago con el del fármaco metformina, el cuál ha sido usado para controlar los padecimientos del sindrome metabólico. Los resultados demostraron que el mucílago tuvo mayor efecto reductor de los parámetros séricos de colesterol y triglicéridos, así como del tejido adiposo, en comparación con el grupo tratado con metformina. También se demostró que el incremento en el nivel de adiponectina fue similar al efecto del fármaco metformina. Otra ventaja del mucílago fue la disminución del daño renal en comparación con el provocado por el fármaco administrado. La caracterización del mucílago mediante FTIR demostró la presencia de grupos funcionales de la familia de los manoglicanos, identificados en el pseudobulbo de otras especies de orquídeas con actividad biológica para reducir parámetros séricos como glucosa, colesterol, triglicéridos, así como obesidad.

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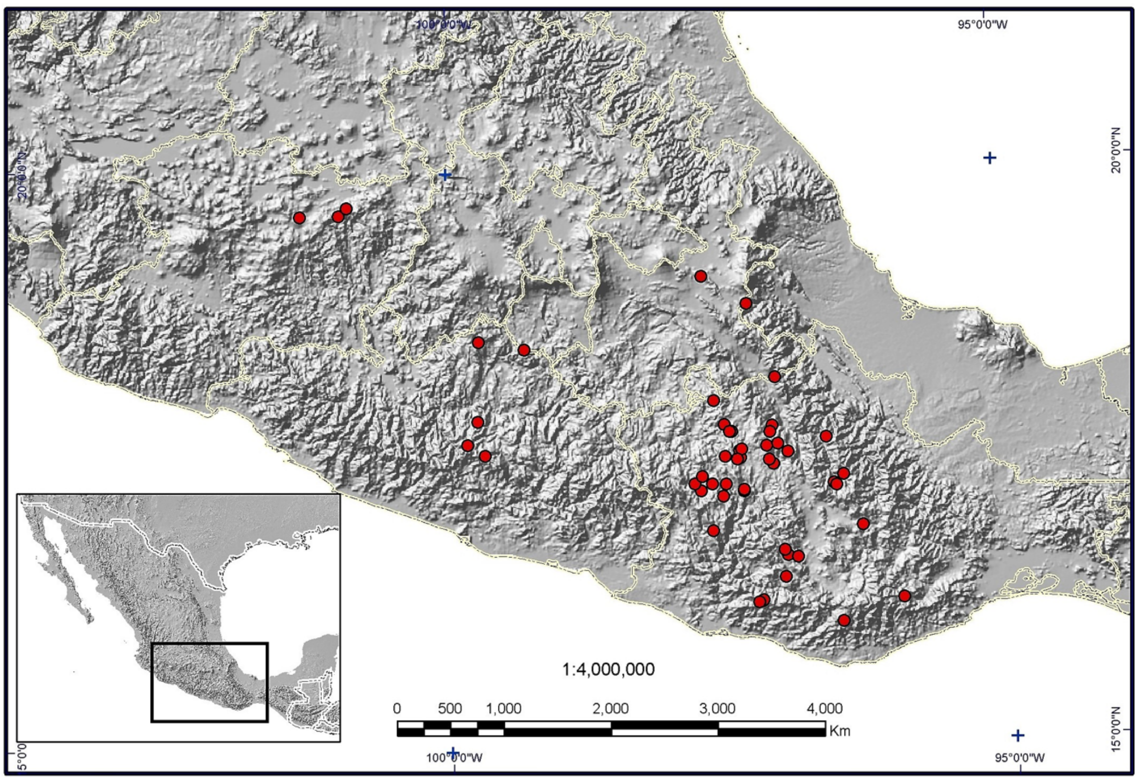

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CAPITULO II

Antioxidant Capacity of *Prosthechea karwi*nskii extracts obtained by sonication

"En la vida no existe nada que temer, sólo cosas que comprender" Marie Curie

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Antioxidant Capacity of *Prosthechea karwinskii* (Orchidaceae) Extracts Obtained by Sonication

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Abstract.

*Prosthechea karwn*skii is a valuable ornamental orchid in Oaxaca, Mexico. In the Mixteca region, this plant has uses in traditional medicine as well as in religious ceremonies. The presence of phenolic compounds with antioxidant potential in this orchid has been reported, although not determined. Considering the advantages of the use of sonication in secondary metabolite extraction from medicinal plants, hydroethanolics and hydroethanolics extracts were obtained from each part of this orchid (pseudobulb, leaf and flower) using sonication pretreatment in order to determine antioxidant capacity. The results indicated that both hydroethanolic (IC50: 3.52 ± 0.078 mg / ml) and hydromethanolic (IC50: 8.88 ± 1.826 mg / ml) leaf extracts had a higher antioxidant capacity than pseudobulbs and flower extracts. Leaf extracts also exhibited inhibition in the breast cancer cell line (MCF-7). The results demonstrated the antioxidant capacity of this orchid and its potential for application in assessments related to oxidative stress diseases.

Key words: antioxidant activity index, cytotoxicity, extraction methods, medicinal plants.

Introduction.

Plant extracts have been widely used in the food and pharmaceutical industries and their quality often depends on the extraction method used to prepare the compounds of interest in terms of antioxidant, antifungal and flavoring properties. In the pharmaceutical industry the solvent extraction of bioactive compounds from plant material is a routine procedure. Recent technological advances and growing medical interest in plant-derived pharmaceuticals, however, have resulted in an increasing importance being placed on more efficient extraction methods that use alternative energy (7,8). Sonication is one of the alternative methods for obtaining secondary metabolites; it favors performance and reduces extraction time. Sonication applies sound energy to produce cavitation bubbles that implode as a result of the shearing force produced in the solvent by the passage of an ultrasonic wave; causing the rupture of the cells, increasing solvent transfer into the sample matrix (or mass transfer) and increasing the contact surface area between the solvent and the secondary metabolites (14,17). Phenolic compounds, one of the most important groups of compounds in plants, have attracted great interest for their ability to stabilize or deactivate free radicals before they attack cells. These compounds are thus able to protect organisms against damage caused by oxidative stress from free radicals (2). *Prosthechea karwinskii* is an ornamental orchid with showy and fragrant flowers. In the Mixteca region of Oaxaca this plant is also used in traditional medicine for the treatment of hyperglycemia (pseudobulb and leaves), coughs (pseudobulb and flowers), burns (pseudobulb) and to minimize the risk of a miscarriage (flowers) (6). A previous study of this orchid identified several phenolic compounds (9) associated with important antioxidant activity, which have not yet been identified in the present species.

In this work the methanolic and ethanolic extracts of pseudobulbs, leaves and flowers from *P. karwinskii* underwent sonication pretreatment in order to evaluate their antioxidant and cytotoxic activity.

Methods.

Plant material and extracts

The plant material was obtained from *Prosthechea karwinskii* specimens previously used as ornaments for Easter celebrations in Zaachila, Oaxaca, with the permission of the organizers of the festivities. A specimen was then entered in the Instituto Politécnico Nacional's Herbarium OAX. The plant material was separated into pseudobulbs, leaves and flowers; each portion was dried, pulverized and stored at room temperature (RT) until use. The hydroethanolic extract (HE) was prepared by placing 10 g of each part of the orchid in 400 ml of an ethanol-deionized water solution (1: 1) for 7 days at RT with constant stirring. The sample was then filtered and evaporated to RT. The hydromethanolic extract (HM) was obtained by placing the sample under reflux for 60 minutes with a methanol-water mixture (70:30). To obtain the extracts with sonication pretreatment prior to the extraction procedure noted above, each sample was sonicated (70W) for 20 minutes. The obtained extracts were evaporated mechanically for 24 hours at RT.

Determination of antioxidant activity index

The antioxidant activity of the extracts was determined by the method of DPPH in a concentration of 0.1 mM according to a modification of the method of Brand et al. (3), the concentration needed to inhibit 50% of free radicals (IC50) was determined, the antioxidant activity index (AAI) was determined as the ratio of the final concentration of DPPH and the IC50 (16).

Data analysis

The results obtained in the determination of total flavonoids and AAI are expressed as the mean ± standard deviation. Data were evaluated using the Minitab 16 software. Statistical significance was determined with an analysis of variance and a Tukey multiple range test was used to test for significant differences between the different extraction methods used. For all analyses the level of significance was $P<0.05$.

Assay of cell growth inhibition

The extracts showing the highest antioxidant activity index were evaluated for cytotoxic activity on six human cancer cell lines: central nervous system glia (U251), prostate (PC-3), leukemia (K562), colon (HCT-15), breast (MCF-7) and lung (SKLU). The cell lines were obtained from the National Cancer Institute (NCI) from the United States of America. The cytotoxicity of the extracts was determined in microcultures, measuring cell viability and growth with the Sulforhodamine B assay according to procedures validated by NCI.

Results.

The results in Table 1 indicate that the *Prosthechea karwinskii* extract with the highest antioxidant activity was the maceration with ultrasound leaf extract (HESleaves) with values of IC_{50} = 3.52 ± 0.078 µg/ml and AAI= 11.2 ± 0.251. These values are higher than those reported for the ethanol-water leaf extract of *Bauhinia kalbreyeri* with AAI 2.1406 (11) and methanolic leaf extract of the orchid *Dendrobium speciosum* with AAI 3.46 * 10^{-5} (10).

Table 1. 50% inhibitory concentration (IC50), antioxidant activity index (AAI) of the hydromethanolic and hydroethanolic extracts of *Prosthechea karwinskii*

Extract	IC50 (mg/ml)	AAI
HESpseudobulb	11.81±0.320	3.33±0.090
HESleaves	**3.52±0.078**	**11.2±0.251***
HESflowers	44.92±3.740	0.79±0.151
HMSpseudobulb	15.46±1.421	2.55±0.233
HMSleaves	**8.88±1.826**	**4.54±0.838**
HMSflowers	24.70±1.381	1.59±0.086
$C_6H_6O_6$	**2.68±0.03**	**14.71±0.165***

$C_6H_8O_6$:ascorbic acid, HE: hydroethanolic extract, HM: hydromethanolic extract, S: sonication. The values represent the mean ± SD, superscript values show statistically significant difference as revealed by the Tukey test (P<0.05).

The HESpseudobulb extract for *P. karwinskii* AAI was 3.33 ± 0.090, higher in comparison to hydromethanolic and chloroform pseudobulb extracts reported for another Mexican orchid, *Prosthechea michuacana* (13) with AAI values of 0.0901 and 0.1268, respectively. For HMSleaves *P. karwinskii* extract, the value of 4.54 ± 0.838 AAI is higher compared with other plant extracts obtained in the same method as in leaves of *Juglans regia*, AAI = 1.98 * 10-9 (4), *Jasminum humile*, AAI=0.5598 (12) and rhizomes of *Curculigo orchioides*, AAI=0.3720 (1).

Table 2. Percent inhibition in cancer cell line

Extract	U251	PC-3	K562	HCT-15	MCF-7	SKLU-1
HESleaves	NE	2.6	NE	7.8	**13.8**	4.8
HMSleaves	NE	NE	NE	6.0	**13.0**	NA

Concentration, (50 µg/ml) vehicle DMSO.U251= central nervous system glia, PC-3= prostate, K562= leukemia, HCT-15= colon, MCF-7=breast, SKLU= lung, NE= no effect.

In Table 2 the percent inhibition results in cancer cell lines are shown. The leaf extracts with antioxidant activity that received sonication pretreatments were more effective in the inhibition of growth in breast cancer cells (MCF7). The antioxidant activity of a sample is considered as an indicator that favors the activation of redox type metabolism in cells where a highly oxidative environment (15) is generated due to cellular inflammation, forming reactive oxygen species. These cause oxidative cell damage generating pro-inflammatory mediators such as cytokines, chemokines and prostaglandins that are converted into angiogenesis initiators, a fundamental process in the malignant transformation of tumor growth and other diseases. Hence, it is understood that at low concentrations of reactive oxygen species, due to the antioxidant effect, the response mechanism against diseases such as cancer, diabetes, arthritis, atherosclerosis, ischemia, immune and endocrine system is enhanced (5,15).

Conclusions.

Sonication promotes plant cell breakage, thus increasing the mass transfer between the solvent and the secondary metabolites and enhancing extracts biological activity. The effect of sonication was higher in the extracts obtained from *Prosthechea karwinskii* leaves as demonstrated in the results of AAI.

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CAPITULO III

Effect of *Prosthechea karwinskii* on obesity and dislipidemia in Wistar rats

"La estabilidad sólo puede lograrse mediante materia inactiva" Marie Curie

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Short Communication

Effect of *Prosthechea karwinskii* (Orchidaceae) on obesity and dyslipidemia in Wistar rats

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A R T I C L E I N F O

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A B S T R A C T

Background: *Prosthechea karwinskii* is an endemic Mexican orchid, it's currently used as decorative element and in the traditional medicine to treat diabetes and some problems related to inflammatory processes.
Aim: To determine antioxidant activity index (AAI) and to validate by the first time and through an rat model the hydroalcoholic extract obtained from *Prosthechea karwinskii*, a plant used in traditional medicine for treat conditions relate to the metabolic syndrome.
Methods: For *in vivo* assays 25 weaned male Wistar rats were divided into a control group (CG; n = 5) and a Metabolic Syndrome group (MS; n = 20). The rats of the latter were induced to MS with 40% sucrose in the drink water during 13 weeks. After MS induction this group was subdivided into 4 groups: MS group (n = 5) received sucrose, and three groups receiving 200 mg/kg of body weight of each extract pseudobulb (P, n = 5), leaf (L, n = 5), and flower (F, n = 5). All treatments were followed for 13 days. Blood was collected at the end of the study to measure glucose, cholesterol and triglycerides. AAI were measured in the extracts by the method of DPPH. The results were analyzed using MINITAB 16.1.0, and the statistical significance was determined by ANOVA and a Tukey's test ($P < 0.05$).
Results: Leaves (L) extract had highest values in AAI, followed by flowers (F) and pseudobulb (P) extracts. Leaves extract had highest reducing effect on glucose level, while flower extract had highest reducing effect on the cholesterol and triglycerides levels.
Conclusions: The *P. karwinskii* extracts evaluated here reduces the glycemic and lipidemic parameters in Wistar rats with MS induced. These effects may be attributed to the high antioxidant capacity of the extracts.

1. Introduction

Medicinal plants play an important role in the introduction of new therapeutic agents as a source of biologically active substances with antihyperlipidemic and antihyperglycemic properties, among other effects.[1] Hyperglycemia results in an increased oxidative stress due to reduced endogenous antioxidants in the body[2] and imbalance in adipose tissue, influencing lipid regulation and triggering cardiovascular disease. Metabolic syndrome (MS) is the term used to designate a set of interrelated conditions that include hyperglycemia, hyperlipidemia, obesity and hypertension.[3–6] In traditional medicine, plants with biological activity affecting metabolic disturbances related to the pathophysiology of MS have been evaluated, including species from Orchidaceae family. Asian orchids such as *Nervilia plicata* (Andrews) Schltr.,[7] *Dendrobium chrysotoxum* Lindl.[8] and *Dendrobium denneanum* Kerr.,[9] have shown to have hypoglycemic effects. In Mexico some orchid species have also been evaluated for conditions related to MS; *Scaphyglottis fasciculate* Hook. for its potential relaxing effect on cardiac contractions,[10] *Laelia autumnalis* (La Llave & Lex.) Lindl. for its antihypertensive effect,[11] *Laelia anceps* Lindl. for its antihypertensive and vasorelaxant effects,[12] and *Prosthechea michuacana* (La Llave & Lex.) W.E. Higgins for its hypoglycemic activity.[13]

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An endemic orchid to the mountains of southern Mexico that is used in the traditional medicine is *Prosthechea karwinskii* (Mart.) J. M.H. Shaw. Different parts of this orchid are used as remedy to treat hyperglycemia (pseudobulb and leaves), cough (pseudobulb and flowers), burns (pseudobulb), and to prevent miscarriages (flowers).[14] The flowering of this species coincides with Easter celebrations and therefore this orchid is also used in religious decorations at homes and churches.[14–16] A previous phytochemical study[17] of our research group has identified the presence of phenolic constituents in this orchid, like tyrosol, apigenin-7-glucoside, caffeic acid, vainillin, *p*-cumaric acid and ferulic acid. These compounds are known by their cardioprotective effects[18,19] due to its ability to inhibit cholesterol oxidation. Furthermore, phenolic compounds can inhibit adipose tissue growth due to their antiangiogenic activity and their ability to regulate adipocyte metabolism.[20]

The goal of this research was to determine the antioxidant activity and to validate, by the first time, el traditional use of *Prosthechea karwinskii* in the treatment of some conditions relate to the metabolic syndrome, for which some parts of the plant are employed (pseudobulb, leaf, or flower). For this, the plant extracts were obtained with ethanol-deionized water and then were evaluated in a rat model.

2. Materials and methods

2.1. Plant material and extracts

Plant material was collected in 2012 and 2013 from specimens that were used as Easter decorations in Villa de Zaachila, Oaxaca. Additional material was collected in San Pedro and San Pablo Teposcolula with the permission of the local authority. Taxonomic determination of the plant was done and pressed specimens were prepared and deposited in OAX Herbarium of the Instituto Politécnico Nacional (Solano 4037). Since in traditional medicine is known that each part of this orchid is used separately for particular condition[14], the plant material was separated into pseudobulbs, leaves and flowers; each portion was dried, pulverized, and stored at room temperature (R.T.) until use. Ten grams of each dried part were placed in 400 ml of ethanol-deionized water solution (1:1, w/v) for 7 days at R.T., frequently stirred, then filtered and concentrated by solvent evaporation at R.T. The yield of the extraction for each plant part was: 18.8% for pseudobulbs, 16.8% for leaves, and 33.6% for flower. The sticky extracts obtained were placed at R.T. until it use.

2.2. Analysis of antioxidant activity index

Antioxidant capacity of the extracts was determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$) method; antioxidant activity index (AAI) and IC_{50} values were determined according to Sherer and Godoy.[21]

2.3. Sucrose-induced metabolic syndrome model

A total of 25 weaned male Wistar rats of 21 days age and 150–200 g body weight (Table 2) were individually housed and maintained in a 12-h light/dark cycle at 25 °C. This study was approved by the Research Committee of the Universidad Cristobal Colón following the guidelines of Mexican legislation, NOM-062-ZOO-1999[22] for the care and use of laboratory animals. Animals were divided into two groups: the control group (CG; n = 5) was given a standard diet (Lab Diet 2004 S, Harlan Teklad Inc.) and water *ad libitum*; the Metabolic Syndrome group (MS; n = 20) which was given the same standard diet plus 40% sucrose in the drinking water *ad libitum* for 13 weeks to induce MS.

2.4. Experimental diet and co-treatment

After MS induction this group (n = 20) was subdivided into 4 groups: MS group (n = 5) received a high caloric sucrose diet, P group (n = 5) received pseudobulb extract, L group (n = 5) received leaf extract, and F group (n = 5) received flower extract; these groups were compared with the control group (CG) as was mentioned above. P, L. and F groups received during 13 days by oral via, through a standard esophageal cannula, 200 mg/kg of body weight of the corresponding evaluated extract dissolved in water. Animals from all groups received the previously mentioned standard diet, during the same period of 13 days; additionally, for the MS group its diet included 40% sucrose in the drinking water *ad libitum*. At the end of the treatment the final weight was registered and a blood sample was taken from each rat with 18-h-fasted using cardiac puncture under anesthetic condition, prior to the killing of the animals. The blood was centrifuged and serum was kept at −20 °C until use.

2.5. Adipose tissue and Biochemical parameters

At the end of the treatment, rats were killed with anesthesia (0.1 ml intraperitoneal of 1% sodium barbiturate) to obtain the abdominal adipose tissue, epididymal and pericardial fat, and to determine the serum levels of glucose, cholesterol and triglyceride. These parameters were determined by enzymatic-colorimetric methods according to the manufacturer's instruction using a biochromatic analyzer model Vitalab Selectra E.

2.6. Data analysis

Data obtained from the serum parameters, weight gain, organs and adipose tissue are presented as the mean ± SD. Data was evaluated using Minitab 16.1.0 software. Statistical significance was determined with an analysis of variance, a Tukey's multiple range test was performed to test for significant differences between the different treatment groups. For all analyses the level of significance was $P < 0.05$.

3. Results

3.1. Total flavonoids and antioxidant activity of Prosthechea karwinskii extracts

Table 1 presents the concentration required to inhibit 50% of free radicals (IC_{50}), antioxidant activity index (AAI), and total flavonoid content of the extracts administered for biological evaluation.

The highest antioxidant capacity was showed by the extract of leaves (AAI = 5.7), followed by that from flowers (AAI = 1.276), and pseudobulbs (AAI = 0.925).

3.2. Caloric intake induced MS model

The daily caloric intake of the MS group compared with that of the control group (CG) during the 13 weeks MS induction period is

Table 1
Antioxidant capacity of *Prosthechea karwinskii* extracts.

Extract	Yield w/w (%)	IC_{50}	AAI
Pseudobulb	18.8 ± 1.335	43.06 ± 7.311	0.925 ± 0.162
Leaf	16.8 ± 2.335	6.91 ± 0.187	5.7 ± 0.157
Flower	33.6 ± 2.93	30.85 ± 2.51	1.276 ± 0.971

IC_{50}: 50% inhibitory concentration. AAI: antioxidant activity index = [DPPH]/IC_{50}.

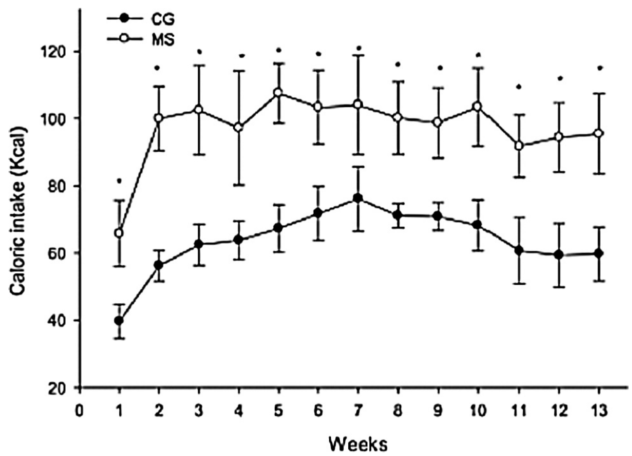

Fig. 1. Caloric intake during Metabolic Syndrome development. CG: control group, MS: sucrose diet, kcal: kilocalories. The values represent the mean ± DS $^{*}P < 0.05$.

shown in Fig. 1. The MS group showed significant differences from the CG due to sucrose consumption.

Table 2 shows the caloric intake of CG, MS, P, L and F groups during 13 days of treatment consisting in the administration of the extracts and sucrose water. The initial weight for each evaluated group was nonuniform because evaluation of extracts began after induction of the animal model metabolic syndrome with 40% sucrose in drinking water; also, it must be considered the variation presented in the caloric intake of the animals during the development of MS, as is shown in Fig. 1.

Body weight of the CG increased by 1.1% while the body weight of the MS group increased by 5.6%. After the treatment, the P group lost the most weight, 7.8% of the total weight. Body weight of the L group decreased by 5.2% whereas that of the F group decreased by 5%, these results were respect to MS group.

3.3. Effect of Prosthechea karwinskii extracts in adipose tissue and serum parameters

Table 3 shows the effect of *P. karwinskii* extracts on abdominal, epididymal and pericardial adipose tissue weight over the 13 days experimental treatment.

In comparison with the MS group, the other groups experienced significant decreases in total adipose tissue: 49% in the P group, 36% in the L group, and 33% in the F group. According to the serum parameters of the CG and the MS group, glucose, cholesterol and triglycerides levels decreased in three groups: P, L and F (Table 4).

The leaf extract (L) had greatest effect in decrease the fasting glucose levels (64.12 mg/dl) in comparison with the MS group (135.26 mg/dl). The other extracts (F and P) also decreased glucose levels to 73.72 and 92.20 mg/dl, respectively. The flower extract (F) had the greatest effect on cholesterol levels (53.89 mg/dl) compared to the MS group (87.52 mg/dl); cholesterol levels in the CG were 69.43 mg/dl. P and L extracts also decreased this parameter but in a lesser extent: 75.55 mg/dl and 76.04 mg/dl, respectively. The extracts also had a reductive effect on triglyceride levels compared to the CG and MS groups, 214.28 and 259.28 mg/dl, respectively. The F group had the lowest triglycerides level (74.58 mg/dl), followed by L (108.78 mg/dl) and P (127.75 mg/dl) groups (Table 4).

4. Discussion

This work reveals by the first time that the pseudobulb, leaf and flower hydroalcoholic extracts from *P. karwinskii* ameliorated key parameters of the MS in a rat model and their relation with antioxidant capacity of the extracts. At the end of the treatment, the group given pseudobulb extract lost the greatest percentage of body weight, followed by the groups that received leaf and flower extracts. The results indicate that the evaluated extracts, administered in a dose of 200 mg/kg/day, significantly decreased adipose tissue mass and serum parameters levels that are associated with MS. The extracts evaluated here, not only reduced the adipose tissue mass but glucose levels as well; in contrast the results reported for aqueous extract from *Ilex* paraguariensis A. St.-Hil.[23] reduced abdominal and epididymal adipose tissue too, but raised glucose levels during 30 days of treatment. As the adiposity index data reveals, *Prosthechea karwinskii* extracts also had a greater effect on reducing adipose tissue than the previously reported for *Salacia reticulate* Wight[24] and *Citrus grandis* (L.) Osbeck.[25]

The extracts also had a lowering effect on cholesterol and triglycerides levels; in this case, the flower extract had a greater effect than the reported for *Camellia sinensis* (L.) Kuntze.[26] Evaluation of *Citrus grandis* hydroalcoholic extract[25] indicates that after two weeks of treatment with a higher administered dose, cholesterol levels remained higher than our results. The dosage of leaf extract evaluated here had a greater glucose lowering effect than that reported for the polysaccharide obtained from *Dendrobium chrysotoxum* pseudobulbs[8] using dosage of 200 mg/kg/day and 500 mg/kg/day. The leaf extract evaluated here also has a better dampening effect than the reported for *Dendrobium denneanum* polysaccharide[9] using a dose of 300 mg/kg/day.

The antihyperglycemic effect of leaf extract in this work could be attributed to its greater antioxidant capacity compared with that for pseudobulb and flower extracts. Since hyperglycemia is associated with diminished endogenous antioxidants and increased oxidative stress, antioxidants have been shown to reduce the risk of hyperglycemia improving glucose disposal in the body.[5,27,28] According to the methodology described by Sherer and Godoy,[21] these results show that *P. karwinskii* extracts have a higher AAI (Table 1) than other species reported in the following studies: ethanol-water leaves extract from *Dendrobium speciosum* Sm.[29] with IAA of $3.46 * 10^{-5}$, the hydromethanolic and chloroform pseudobulb extracts from *Prosthechea michuacana* with an AAI of 0.0901 and 0.1268, respectively,[13] the hydrometanolic rhizomes extract from *Curculigo orchioides* Gaertn.[30] with an AAI = 0.3720, the hydrometanolic leaf extract of *Juglans regia* L.[31] with an AAI = $1.98 * 10^{-9}$, and *Jasminum humile* L.[32] with an AAI = 0.5598.

Table 2
Caloric intake and body weight of rats under the effect of *P. karwinskii* extracts after 13 days of experimental treatment.

Parameter	CG	MS	P	L	F
kcal/day (B)	65.10 ± 19.53	125.68 ± 22.62[a]	87.43 ± 2.38	91.27 ± 10.38	81.30 ± 9.26
Initial weight (g)	372.0 ± 70.14	440.0 ± 83.19	321.67 ± 96.3	385.0 ± 69.28	431.67 ± 23.63
kcal/day (E)	79.05 ± 6.48	139.42 ± 6.77[a]	128.35 ± 6.47[a]	147.93 ± 31.13[a]	147.78 ± 13.96[a]
Final weight (g)	376.0 ± 77.73	464.50 ± 44.04	296.67 ± 88.8	365.0 ± 73.65	410.0 ± 20.00

CG: control group, MS: sucrose diet, P: sucrose diet and pseudobulb extract, L: sucrose diet and leaf extract, F: sucrose diet and flower extract, B: Beginning of the treatment, E: End of the treatment. The values represent the mean ± SD, superscript values show statistically significant difference as revealed by the Tukey's test.
[a] $P < 0.05$.

Table 3
Abdominal, epididymal and pericardial adipose tissue weight and adiposity index after co-treatment with *P. karwinskii* extracts.

Parameter (g)	GC	MS	P	L	F
AT abdominal	12.20 ± 1.92	16.25 ± 5.91	9.33 ± 3.78[a]	11.66 ± 2.30	11.0 ± 1.73[a]
AT epididymal	10.0 ± 2.55	15.83 ± 6.67	6.66 ± 2.08[a]	8.33 ± 2.88[a]	10.33 ± 0.57[a]
AT pericardial	0.62 ± 0.40	0.48 ± 0.18	0.70 ± 0.26	0.56 ± 0.30	0.40 ± 0.10
Total fat	22.82 ± 4.87	32.56 ± 12.76	16.69 ± 6.12[a]	20.55 ± 5.48[a]	21.73 ± 2.4[a]

AT: adipose tissue, GC: control group, MS: sucrose diet, P: sucrose diet and pseudobulb extract, L: sucrose diet and leaf extract, F: sucrose diet and flower extract. The values represent the mean ± SD, superscript values show statistically significant difference as revealed by the Tukey's test.
[a] $P < 0.05$.

Table 4
Variation of serum parameters in Wistar rats after 13 days of treatment with *P. karwinskii* extracts.

Parameter (mg/dl)	CG	MS	P	L	F
Glucose	96.80 ± 10.64[a]	135.26 ± 30.70	92.20 ± 7.95[a]	64.12 ± 11.39[a]	73.72 ± 25.76[a]
Cholesterol	69.43 ± 10.39[a]	87.52 ± 7.44	75.55 ± 0.32[a]	76.04 ± 9.59[a]	53.89 ± 10.54[a]
Triglycerides	214.28 ± 18.07[a]	259.28 ± 23.89	127.75 ± 46.56[a]	108.78 ± 11.30[a]	74.58 ± 22.35[a]

CG: control group, MS: sucrose diet, P: sucrose diet and pseudobulb extract, L: sucrose diet and leaf extract, F: sucrose diet and flower extract. The values represent the mean ± SD, superscript values show statistically significant difference as revealed by the Tukey's test.
[a] $P < 0.05$.

Furthermore, this study confirms the potential antioxidant activity of compounds previously identified in *P. karwinskii* by Mijangos-Ricardez and López-Luna.[17]

Polyphenolic compounds also have been considered as a potential alternative for the treatment of MS given their effect in the absorption and metabolism of simple carbohydrates,[18,33] mainly reflected in the hypoglycemic and hypolipidemic effect of the extracts evaluated in this work. Recent studies have established that phenolic and flavonoid compounds are both capable of inhibiting lipid accumulation and apoptosis induction,[34] although they regulate the adipocyte physiology in a different way. An hypercaloric diet and genetic predisposition are the immediate causes of developing MS risk factors, such as obesity, insulin resistance, hyperlipidemia and hypertension, as well as of other metabolic diseases such as type II diabetes and cardiovascular disease.[35] In this work the hypercaloric diet (40% sucrose solution) administered at the beginning of the experiments was the principal factor who caused MS in the rats, thereby this work demonstrates that the extracts herein evaluated decreased the adiposity index and thus its relationship with associated metabolic disorders (hyperglycemia, hypercholesterolemia and hypertriglyceridemia).

Prosthechea karwisnkii extracts, by reducing adipose tissue, may also decrease characteristics MS serum parameters. This is because adipose tissue regulates the activation of macrophages, which favor the secretion of adipokines that regulates insulin resistance and the accumulation of triglycerides and cholesterol caused by increase of adiponectin and leptin secretions, in order to maintain equilibrium with the excess nutrients consumed by the organism.[34,35] For this reason, is require carry out studies that allow to evaluate the effect of the extracts on adipose tissue and dyslipidemia in a MS rat model, this research are actually in progress. In conclusion, the hydroalcoholic extracts studied hereof represent an alternative for the treatment of MS in the traditional medicine. Their reductive effect was showed even with the continued administration of a hyper-caloric diet to the groups treated with the extracts.

Conflict of interest statement

The authors declare that this article content has no conflicts of interest.

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CAPITULO IV

Kidney protection and hypolipidemic effect of *Prosthechea karwinskii* (Orchidaceae) mucilage in Wistar rats

"La Ciencia es el instrumento de Dios para demostrar a la humanidad todas sus bendiciones" A.R.O.

Article title: Kidney protection and hypolipidemic effect of *Prosthechea karwinskii* (Orchidaceae) mucilage in Wistar rats

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Abstract

Introduction: *Prosthechea karwinskii* is an endemic Mexican orchid used in traditional medicine for treatment of diabetes and other conditions; the effect on obesity and dyslipidaemia of hydroalcoholic extracts of this plant, attributed to its antioxidant capacity, has been reported previously. In this work, the effect of mucilage extracted from pseudobulbs was evaluated for its effect on adiponectin, adipose tissue, cholesterol, creatinine, triglycerides, urea and uric acid levels. **Methods:** 25 Wistar rats were divided into a healthy control group (n = 5), and a metabolic syndrome (MS) group (n = 20) that was induced to MS with 40% sucrose-diet over 13 weeks. The MS group was divided into four groups of n = 5: a control group, a positive control group that received metformin 200 mg/kg/day, a group that received mucilage extract at a dosage of 200 mg/kg/day and sucrose-diet, and a group that received mucilage extract at a same dosage without sucrose-diet. After 13 days of treatment the animals were sacrificed to obtain blood to perform serum analyses and to evaluate the kidney and adipose tissue. The mucilage was purified and identified by FTIR. **Results:** The extract evaluated showed protective renal effects due to the absence of glomerular atrophy; renal haematuria was less severe in rats treated with mucilage. There was an increase in adiponectin levels in rats treated with mucilage, which was correlated with a significant decrease in cholesterol and triglyceride parameters. **Conclusion:** The present work demonstrated the potential use of this mucilage in the treatment of MS and kidney disorders associated with that pathology.

Key words: Adiponectin, creatinine, urea, uric acid, adipose tissue, polysaccharide.

INTRODUCTION

Metabolic syndrome (MS) is the term used to describe the group of interrelated conditions that include obesity, hyperglycaemia, hyperlipidaemia and hypertension[1–4] caused by the ingestion of a hypercaloric diet. The greatest risk factor for this condition is the increase in adipose tissue (AT) due to the lack of control of regulatory adipokines such as adiponectin, a hormone produced in adipose tissue. When the level of adiponectin decreases, renal pathophysiology and arteriosclerosis develop[5]. When AT expands its volume, it can lead to complications at a systemic level. This effect is reflected in the decrease of plasma adiponectin concentration in AT, which is one of the most abundant adipokines secreted by AT[6,7,8]. Some authors[9,10] have shown that there is a relationship between levels of adiponectin and uric acid that are related to MS conditions.

It has been discovered that some complex groups of indigestible compounds present in the small intestine, including complex polysaccharides, hemicelluloses, oligosaccharides, gums, pectins and carboxylic acids, have been effective in regulating the parameters related to MS, such as body weight, lipid profile, hypertension and glucose homeostasis, because they are partially or fully fermented in the large intestine and colon[4,5]. Studies on Asian *Dendrobium* orchids reported the presence of complex polysaccharides in pseudobulbs of species such as *Dendrobium denneanum*[11]*, D. nobile*[12–14], *D. officinale*[15–17], *D. fimbhriatum*[13], and *D. densiflorum*[18], which have a stimulating effect on the immune system, an antioxidant or hypoglycaemic function, and in decreasing obesity.

Prosthechea karwinskii is an endemic Mexican orchid. The use of this species in traditional medicine has been reported for the treatment of diabetes and other conditions[19]. Results analysed previously by Rojas-Olivos et al.[20] have demonstrated that hydroalcoholic extracts from pseudobulbs, leaves and flowers of *P. karwinskii* have an effect on obesity and dyslipidaemia, using an animal model. However, the extract that showed the greatest effect on the reduction of AT was that obtained from the pseudobulb.

Given the above-mentioned results, the aim of this study was to determine the effect of the mucilage of the pseudobulb of *P. karwinskii* on renal protection, AT and dyslipidaemia to compare it with the results obtained from the effect of metformin, and to characterise the mucilage by FTIR to identify functional groups related to its biological activity.

METHODS

Obtaining pseudobulb extract (mucilage)

Dehydrated pseudobulbs (180 g) from adult *P. karwinskii* plants were macerated with 1:1 v/v ethanol-water for 7 days at room temperature. The extract obtained was filtered and then concentrated, dissolved in deionised water in a proportion of 1:10 w/v to obtain a concentrated aqueous extract, which was mixed with four volumes of ethanol under constant stirring for 10 min. The precipitate obtained (mucilage) was redissolved in water and deproteinated with Sevag reagent five times consecutively. It was maintained at room temperature until use.

Sucrose-induced metabolic syndrome (MS) model

A total of 25 weaned male Wistar rats, 21 days of age and 150–200 g in weight, were individually housed and maintained in a 12-h light/dark cycle, at 25 °C. This study was approved by the Research Committee of the Universidad Cristobal Colón following the guidelines of Mexican legislation, NOM-062-ZOO-1999[21] for the care and use of laboratory animals, and according to the National Institute of Health Guide for Care and Use of Laboratory Animals, USA[22].The rats were divided into two groups: the control group (C; n = 5) received a standard diet (Lab Diet 2004 S, Harlan Teklad Inc.) and water *ad libitum*; the Metabolic Syndrome group (MS; n = 20) received the same standard diet plus 40% sucrose in drinking water *ad libitum* for 13 weeks to induce MS.

Experimental diet and co-treatment

After MS induction, this group was subdivided into four groups: the MS group (n = 5) received a high caloric sucrose diet (SD); the PKPS group (n = 5) received 200 mg/kg/day mucilage and SD; the PKP group (n = 5) received 200 mg/kg/day mucilage without SD; and the MET group (n = 5) received 200 mg/kg/day metformin and SD. These groups were compared with the control group (C) as mentioned above. All the groups received the treatment over 13 days by oral route, through a standard oesophageal cannula, as well the dose of 200 mg/kg of body weight of the corresponding evaluated extract dissolved in water.

Biochemical parameters and AT

At the end of the treatment, the rats were killed by decapitation to obtain the abdominal AT, and epididymal and pericardial fat. The levels of adiponectin, cholesterol and triglyceride were determined from the blood serum by enzymatic-colorimetric methods, according to the manufacturer's instructions, using a biochromatic analyser model

Vitalab Selectra. The level of adiponectin was assessed in the blood serum using the Adiponectin EIA kit (Sigma-Aldrich).

Statistical analysis

Data obtained from the serum parameters, differences in body weight, and AT are presented as the mean and its standard deviation (SD). Statistical differences between treatments (groups) were determined with analysis of variance and a Dunnett's multiple range test. For all analyses, the significance level was $P < 0.05$. Statistical analyses were performed using Minitab 17 software (www.minitab.com).

Histological analysis

After the animals were sacrificed, a portion of the kidney was fixed in 10% formaldehyde. An Olympus BX51 microscope equipped with a C3040ZOOM digital camera (Olympus, America Inc., Corporate Center Drive, Melville, NY, USA) image analyser was used with a magnification of 20× to compare the effects of metformin and the extract evaluated.

Purification of *P. karwinskii* mucilage

The extract from *P. karwinskii* was precipitated again using ethanol 1:4 v/v, then dissolved in water[15,23] in order to be introduced into a 15 × 550 mm DEAE-Cellulose column, which was previously equilibrated with distilled water[11]. The mobile phases of the column were 200 mL of distilled water (PKPW), 200 mL of NaCl 0.05 M (PKP1), 200 mL of NaCl 0.1 M (PKP2), 200 mL of NaCl 0.2 M (PKP3), 200 mL of NaCl 0.4 M (PKP4), 200 mL of NaCl 0.8 M (PKP5). The flow of the column was 1 mL/min, and the fractions were collected to 8 mL. The elution profile obtained during the purification of the polysaccharide from *P. karwinskii* mucilage was determined by the total carbohydrate content by the phenol–sulphuric acid method from each fraction obtained. One major fraction was selected, obtained during the elution phase with H_2O, and analysed using an FTIR-ATR spectrometer (Thermo Scientific Nicolet 6700).

RESULTS

Post-treatment effect of *P. karwinskii* mucilage on serum parameters

The effects on the rats' serum parameters (adiponectin, cholesterol, creatinine, triglycerides, urea and uric acid) at the end of the treatments are shown in Table 1. The group fed only with the orchid polysaccharide (PKP) showed a significant difference in cholesterol and triglyceride levels compared to the sick (MS) and healthy control (C) groups. The group that received metformin (MET) showed a significant difference only in triglyceride levels compared to both the sick (MS) and healthy control (C) groups.

The serum adiponectin level was higher in the PKP group, related to the effect of the extract without SD. The results related to renal pathology demonstrated that creatinine and urea levels, registered in the groups administered with metformin and orchid mucilage (MET and PKP, respectively), were significantly lower compared to the sick group (MS).

The results of the effect on the amount of AT due to the ingestion of orchid extracts and metformin are shown in Table 2. A significant difference was evident between the groups treated with orchid mucilage without SD (PKP) and the sick group (MS). In addition, only the PKP and MET groups showed a difference between the initial and final weights of the rats during the treatment. This was demonstrated by the adiposity index, which considers the relationship between the amount of AT (total fat) and the final weight at the end of treatment.

Kidney histopathology

Figure 1 shows the histopathology of the rat kidneys after the treatments. The group administered with only the orchid polysaccharide, without SD, suffered less renal damage compared to the group that received metformin (MET).

In the PKP group, haematuria and glomerular atrophy did not appear as severe as in the MS (Fig. 1 A and B); and in the PKP group the Bowman's capsule was not lost as in the MET group (Fig. 1 C and D).

Mucilage purification and analysis of the majority fractions by FTIR

The FTIR spectra of the major fractions are represented in Figure 2. The fractions exhibited two characteristic bands: the first, around 3429 cm^{-1}, corresponds to a hydroxyl group, while the second at 2929 cm^{-1} represents a C–H bond. The band between 1627 and 1651 cm^{-1} is due to a water bond. This polysaccharide fraction has a characteristic band in the region between 1200 and 1000 cm^{-1}; this region is dominated by ring vibrations (C–OH) next to the glycosidic bonds (C–O–C)[23]. A characteristic band around 872 cm^{-1} was found in the major-fraction, indicating the beta configuration of sugar units due to the presence of mannose and glucose[24]. The description of the functional groups mentioned above shows that the composition of the mucilage corresponds to the family of mannoglycans.

DISCUSSION

An increase in the level of adiponectin decreases the risk of developing chronic kidney disease and also regulates lipid levels, as well as other conditions associated with MS. In this work, it is shown that the mucilage of *P. karwinskii*, characterised as a mannoglycan, is related to the increase in adiponectin and affects serum parameters linked to renal protection, also causing a hypolipidaemic effect[5,9,25,26]. The analysis presented here demonstrates that the mucilage belongs to the family of mannoglycans, and the compounds present in it coincide with those reported for other species of orchids[23,24]. On the other hand, the polysaccharide evaluated here showed similar effectiveness for characteristic parameters of MS in comparison to metformin, with respect to adiposity index and levels of creatinine, triglycerides and urea. But unlike this drug, the polysaccharide present in orchid mucilage prevents kidney damage, as was revealed by the histological analysis of this organ. Haematuria decreased in animals that consumed the polysaccharide from *P. karwinskii*, while in animals to which metformin was administered, kidney haematuria was a negative secondary effect. This benefit of *P. karwisnkii* mucilage on the kidney may be attributed to the antioxidant activity previously reported by oxidative stress reduction[20].

The immediate factors causing MS are hypercaloric diet and genetic predisposition, generating several effects, such as obesity, insulin resistance, hyperlipidaemia and hypertension. These factors are the basis for the development of pathologies such as type II diabetes and cardiovascular diseases. The major sources of secretion of proinflammatory cytokines, such as adiponectin, are the AT and the immune system. The invasion of AT by immune cells (macrophages) is the result of their inability to metabolise the continuous flow of complex carbohydrates, due to the insulin resistance generated and the disordered secretion of cytokines from adipocytes present in AT[27,28].

AT is a key condition in the development of MS, because the cells that accumulate an excess of circulating energy, which is provided by a diet rich in simple carbohydrates and saturated fats, cause an inadequate metabolic response to the overload of nutrients that cannot be absorbed, handled or eliminated[6,7,29,30]. The possible mechanism by which *P. karwinskii* mucilage provokes a decrease in AT and the characteristic MS serum parameters may be the regulation of macrophage activation by the AT, which favours the secretion of adipokines that regulate insulin resistance as well as triglyceride and cholesterol accumulation. It is also important to note that the extract of *P. karwinskii* has a reducing effect on AT. In addition, studies have shown that other species of orchids stimulate the activity of the immune system[15,17,18,31,32].

It is possible to attribute the reductive effect of *P. karwinskii* on parameters associated with MS to the presence of polysaccharides, such as mannoglycans, present in this plant, as the FTIR analysis has shown in the present work. These types of compounds have been reported in other studies to have hypoglycaemic and hypolipidaemic effects[29,33,34]. Moreover, as these polysaccharides are soluble fibres, two possible mechanisms of action may be suggested: one is the production of short-chain fatty acids (such as acetate, butyrate and propionate), due to their fermentation in the colon, which is also associated with gluconeogenesis and the regulation of lipid levels[35]. The second possible mechanism of action is the ability of the soluble fibres to retard the absorption of simple carbohydrates in the small intestine, decreasing insulin requirements for glucose and, in the case of lipid-soluble fibre, fixing minerals required to facilitate lipid digestion[28]. Another possible mechanism, by which the extracts of *P. karwinskii* favour the decrease in AT and reduce the characteristic serum parameters of MS, is that this tissue regulates the activation of macrophages through the action of adiponectin and the stimulation of adipokine secretion, which regulates insulin resistance[3,4]. In this work we showed that *P. karwinskii* mucilage reduces the levels of creatinine, urea and uric acid, demonstrating a greater kidney protective effect compared to metformin. It also increases the adiponectin level and reduces AT, factors that favour dyslipidaemia and the reduction of cholesterol and triglyceride levels. The presence of mannoglycans identified by FTIR corroborates the potential use of this mucilage for future work in the treatment of MS and kidney disorders associated with MS pathology.

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Conflicts of interest

The authors declare no conflicts of interest.

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TABLE 1: Variation of serum parameters in Wistar rats after 13 days of treatment with *Prosthechea karwinskii* mucilage (n=5) (mean± SD)

Parameter (mg/dl)	C	MS	MET	PKP	PKPS
Cholesterol	129.87±3.94	152.19±5.64[b]	126.49±34.42	101.97±7.68[a,b]	161.76±12.55
Triglicerides	89.5±3.18	110.5±4.59[b]	57.81±10.77[a,b]	64.25±19.5[a,b]	115±9.86
Adiponectin	0.93±0.04	0.86±0.01[b]	0.88±0.02	0.90±0.02	0.86±0.03
Creatinine	0.81 ± 0.05	0.97±0.09[b]	0.79±0.05[a]	0.84±0.02[a]	0.78±0.04[a]
Uric acid	2.15±0.05	2.92±0.2[b]	2.36±0.14	2.08±0.05[a]	1.87±0.11[a]
Urea	40.6±0.62	42.64±0.58[b]	40.31±0.86[a]	39.78±0.57[a]	44.7±0.5[b]

Data represent means of the experiments performed with n=5. Means followed by "**a"** are significantly differente at *P*<0.05 compared to group MS. Means followed by **"b"** are significantly different at *P*<0.05 compared to group C. **C**, control; **MS**, sucrose diet; **MET**: metformin 200mg/kg; **PKP**, mucilage 200mg/kg; **PKPS**, sucrose diet and mucilage 200mg/kg.

TABLE 2: Abdominal, epididymal, pericardial adipose tissue weight and adiposity index after co-treatment with *Prosthechea karwinskii* mucilage (n=5) (mean± SD).

Parameter (g)	C	MS	MET	PKP	PKPS
AT abdominal	6.6±0.54	10 ± 2.82^{b}	6.94 ± 1.88^{a}	5.42 ± 0.33^{a}	9.1 ± 2.64^{b}
AT epididymal	4.5±0.5	7.2 ± 1.78^{b}	6.32 ± 2.49^{b}	4.2 ± 0.83^{a}	6.68 ± 1.44^{b}
AT pericardial	0.27±0.09	0.66 ± 0.21^{b}	0.6 ± 0.31^{b}	0.24 ± 0.04^{a}	0.95 ± 0.20^{b}
Total fat	11.37±1.13	17.86±4.81	13.86±4.68	9.86±1.2	16.73±4.28
Inicial weight	455±53.38	369±39.42	418.6±60.8	437.5±2.05	398.6±40.05
Final weight	416.4±44.48	351±34.55	383.6±64.74	402±12.54	366.2±46.41
Adiposity index	2.73	5.08^{b}	3.61^{a}	2.45^{a}	4.56

Data represent means of the experiments performed with n=5. Means followed by "**a"** are significantly differente at *P*<0.05 compared to group MS. Means followed by **"b"** are significantly different at *P*<0.05 compared to group C. **AT**, adipose tissue; **C**, control; **MS**, sucrose diet; **MET**: metformin 200mg/kg; **PKP**, mucilage 200mg/kg; **PKPS**, sucrose diet and mucilage 200mg/kg. **Adiposity index**= (Total fat / final weight)*100.

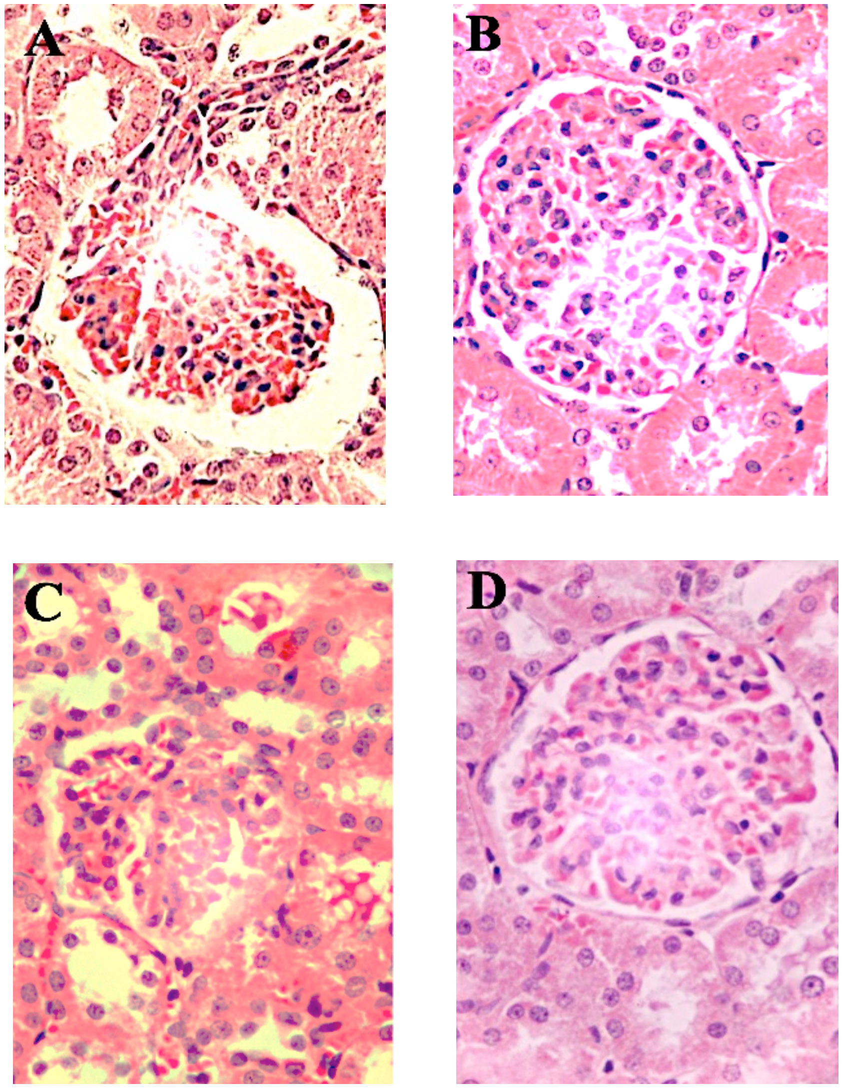

Figure 1- Kidney histophathology from Wistar rats of the control and experimental groups A: glomerular atrophy (MS group), B: normal glomerular zone (C group), C: Loss of Bowman's capsule (MET group), D: encapsulated renal glomerulus (PKP group).

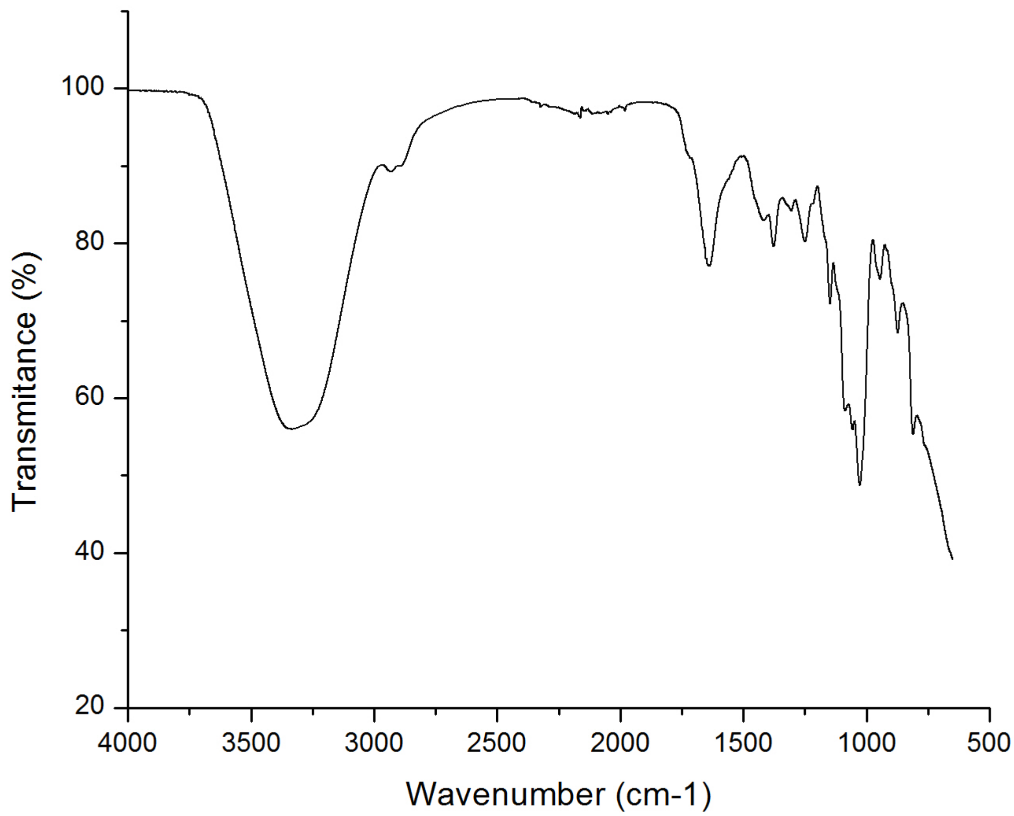

Figure 2- FT-IR spectrum of the major fraction from mucilage obtained from Prosthechea karwisnkii pseudobulbs.

CONCLUSIONES GENERALES Y PERSPECTIVAS

Los resultados de este trabajo dirigido a obtener los extractos de cada parte de la orquídea *Prosthechea karwisnkii*, caracterizar su capacidad antioxidante y evaluar su efecto en los parámetros característicos del síndrome metabólico, han permitido concluir que:

La capacidad antioxidante de los extractos esta relacionada con el contenido de flavonoides totales y el método de extracción. Debido a lo anterior el extracto hidroetanólico e hidrometanólico de la hoja tuvo mayor contenido de flavonoides y un efecto inhibitorio mayor en líneas celulares de cáncer de mama, además los extractos de seudobulbo y flores, presentaron una menor capacidad antioxidante y no presentaron un efecto inhibitorio en ninguna línea celular de cáncer.

Respecto a la evaluación biológica en modelo murino con síndrome metabólico inducido, dónde fueron evaluados los extractos hidroetanólicos de hoja, flor y sedubulbo, el extracto de hoja demostró mayor efecto reductor en el nivel de glucosa, mientras que el extracto de flores tuvo un efecto reductor mayor de los niveles de colesterol y triglicéridos. El extracto de sedubulbo redujo significativamente el tejido adiposo; este es un resultado importante, pues en este tejido sucede la regulación de macrófagos, los cuáles favorecen la secreción de citocinas que regulan la resistencia a la insulina, así como la acumulación de colesterol y triglicéridos causado por el incremento de adiponectina y leptina.

Debido a lo anterior, al evaluar el mucílago obtenido del pseudobulbo, éste demostró un efecto reductor del tejido adiposo y un aumento en el nivel de adiponectina; además, el mucílago tuvo un efecto protector del riñón como lo indican los cortes histológicos del modelo murino evaluado, así como una reducción en los parámetros de creatinina, ácido úrico y urea. El análisis por FTIR del mucílago muestra la presencia de grupos funcionales característicos de la familia de los manoglicanos; para este tipo de compuestos se ha reportado su efectividad en distintos padecimientos del síndrome metabólico donde han corroborado su efecto en retardar la absorción de carbohidratos en el intestino delgado y por lo tanto limita el incremento del tejido adiposo.

Los objetivos alcanzados en esta investigación forman parte del primer reporte de evaluación de la actividad biológica de la especie *Prosthechea karwinskii* (Orchidaceae) dónde se ha corroborado el uso de la orquídea en medicina tradicional para el control de la diabetes, pero se han identificado otros usos potenciales de esta planta como los mencionados anteriormente, planteando nuevas hipótesis que fortalecerán la investigación acerca de esta especie con potencial de aprovechamiento etnobotánico sustentable; tales como el hecho de considerar el aislamiento de los principios activos de cada extracto para ser evaluados durante periodos prolongados de tiempo en el mismo modelo animal inducido a SM y realizar combinaciones de los métodos de extracción convencionales con microondas o ultrasonido para aumentar el rendimiento de los principios activos identificados o bien considerar otras evaluaciones in vitro o in vivo relacionadas con el índice de actividad antioxidante reportado en esta investigación, otra perspectiva importante a considerar sería la síntesis de los principios activos identificados y así considerar al aprovechamiento del potencial farmacológico de *Prosthechea karwinskii* sin afectar las poblaciones endémicas de la especie. Es importante resaltar que el aprovechamiento de los beneficios de las plantas medicinales no debe confundirse con promover el consumo descontrolado de esta especie, afectando las poblaciones en su hábitat. Para evitar esto es importante considerar el desarrollo de estrategias multidisciplinarias para implementar proyectos que favorezcan la disposición sostenible de las especies de orquídeas medicinales en las comunidades, así como el manejo responsable de la información obtenida en artículos científicos.