Centro de Investigación en Alimentación y Desarrollo, A.C.

POTENCIAL ANTI-INFLAMATORIO DE EXTRACTO Y MICROCÁPSULAS DE ORÉGANO (*Lippia graveolens*) SOBRE MODELOS DE INFLAMACIÓN INDUCIDA EN RATONES

Por:

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TESIS APROBADA POR LA

COORDINACIÓN DE CIENCIA Y TECNOLOGÍA PARA PRODUCTOS AGRÍCOLAS DE ZONAS TROPICALES Y SUBTROPICALES

Como requisito parcial para obtener el grado de

DOCTOR EN CIENCIAS

Culiacán, Sinaloa Enero 2025

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Los miembros del comité designado para la revisión de la tesis de Manuel de Jesús Bernal Millán, la han encontrado satisfactoria y recomiendan que sea aceptada como requisito parcial para obtener el grado de Doctor en Ciencias.

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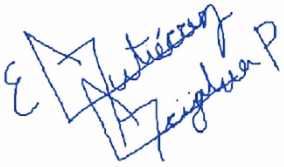

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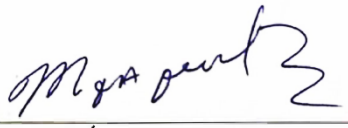

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AGRADECIMIENTOS

A la Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI) por el apoyo económico otorgado durante mis estudios.

Al Centro de Investigación en Alimentación y Desarrollo, AC (CIAD), por darme la oportunidad de formar parte de su institución.

A mi director de tesis, Dr. José Basilio Heredia por siempre confiar en mí y darme la oportunidad de seguir siendo parte del LAFN, por tener siempre las puertas abiertas para cualquier situación, por todo su apoyo y paciencia.

A mi codirectora de tesis, Dra. Miriam Carrasco Portugal por todo su apoyo en este proyecto. Por cobijarme durante mi estancia en CDMX, por siempre preocuparse por mi bienestar, además de brindarme su amistad.

A los miembros del comité de tesis, Dra. Josefina León, Dr. Erick Gutiérrez y Dr. Miguel Angel Angulo por toda la asesoría brindada, las sugerencias acertadas, la confianza y su buena disposición en todo momento durante la realización del proyecto.

A mi Doctora favorita, mi segunda madre, gracias por todo lo que me ha enseñado, por sus acertados consejos, su apoyo y su cariño sincero. Mi más sincero respeto y admiración para usted, la aprecio y la quiero mucho mi maestra favorita por siempre Laura.

A todos los integrantes del laboratorio de farmacología clínica y experimental del INER, Dr. Francisco Flores, Dr. Eduardo Roa, Dr. Juan Carlos Huerta, Dr. Juan Silverio Rodríguez y Marysandra Hernández, muchas gracias por todo su apoyo y disposición para sacar adelante el proyecto.

Al Dr. Eduardo Roa un agradecimiento especial, por ser una pieza fundamental para el desarrollo de este proyecto, por toda su ayuda técnica y académica para el montaje de los experimentos, por siempre estar dispuesto a ayudarme y nunca dejarme solo.

A mis amigos de CDMX, Roberto (chino), José Antonio (Pepe), Fernando, Rachel, gracias por su amistad durante mi estancia, por todos los buenos momentos que compartimos. Siempre tendrán un amigo con quien contar.

A todos los integrantes del LAFN: Dra. Nayeli, Dra. Marilyn, Dra. Melissa, Dr. Luis Ángel, Alicia, Aurelio, Rigoberto, Alejandra y Saraí por su apoyo y amistad durante esta etapa.

A mis compañeros de generación, Magdalena, Alicia, Aurelio, Dulce y Ana Karina por compartir su tiempo y amistad.
A mis amigos Olimpia, Majo, Lalo, Lucí, Brandon y Yudit, por su amistad y los buenos momentos que pasamos juntos.
A mis padres Fernanda Millán y José Luis Bernal por su apoyo incondicional, por creer en mí y tenerme esa confianza total, gracias por permitirme soñar, impulsarme a cumplir mis metas, jamás los defraudaré; gracias por su inmenso cariño.
A mis hermanos Damián, Oscar, Anabel y a mis sobrinos Lupita, Marely, Eduardo, Johana y MariFer por todo su cariño, su amor puro y sincero, por todos los momentos felices que me hacen pasar, los quiero mucho.
A mi novia y compañera de vida Daizha, por todos los buenos momentos que pasamos juntos, por siempre confiar en mí, motivarme a seguir adelante, por siempre hablarme lindo, por compartirme su amor y cariño.
Finalmente gracias a todas las personas que de alguna manera han sido participes en mi formación académica y personal.

DEDICATORIA

A mi madre Fernanda, la luz de mis ojos, el ser más importante de mi vida, gracias por enseñarme el buen camino e impulsarme a luchar por todos mis sueños.

Te Amo Mamá.

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RESUMEN

La inflamación es el proceso por el cual el sistema inmunológico reconoce y elimina los estímulos dañinos y extraños. Durante la inflamación, algunas células promueven la producción de mediadores proinflamatorios como las interleucinas IL-1β, IL-6, IL-8, TNF-α y ROS, entre otros. La sobreproducción de estos mediadores se ha relacionado con la aparición de enfermedades crónico-degenerativas. Para su tratamiento se usan medicamentos antiinflamatorios esteroideos y no esteroideos. Sin embargo, el uso de estos genera efectos secundarios. Como alternativa y coadyuvante en el tratamiento de la inflamación, desde hace décadas se ha utilizado la planta de orégano, ya que se ha reportado que contiene compuestos bioactivos (flavonoides) a los cuales se les han atribuido propiedades antiinflamatorias. Sin embargo, una vez extraídos, los flavonoides deben ser rentables, es decir, eficaces contra enfermedades y de larga vida en procesos de almacenamiento, para ello se emplea la microencapsulación. Por todo lo anterior, el objetivo de este proyecto es evaluar el potencial antiinflamatorio de flavonoides en extracto y microcápsulas de orégano (*Lippia graveolens)* sobre un modelo de ratones con inflamación inducida con carragenina y ovoalbúmina. Para ello, se obtuvieron extractos mediante diferentes métodos de extracción (maceración, extracción asistida por ultrasonido y extracción por fluido supercrítico). Se evaluó el contenido de compuestos fenólicos totales, flavonoides totales, capacidad antioxidante por diferentes métodos (ABTS, DPPH, FRAP y ORAC). También se identificaron y cuantificaron los flavonoides en cada extracto por UPLC-TQS-MS/MS y se llevó a cabo la microencapsulación del extracto. Se indujo el edema en la pata de los ratones con carragenina, mientras que se sensibilizó y se expuso al asma alérgica con ovoalbúmina. Se administraron muestras de *L. graveolens* por vía oral a ratones para evaluar el tamaño del edema. Se analizó el recuento de células inflamatorias, el nivel de IgE sérica y la detección de los niveles de citocinas proinflamatorias en suero, tejido plantar y tejido pulmonar. Se identificaron y cuantificaron 14 flavonoides. Los tratamientos con *L. graveolens* redujeron significativamente el tamaño del edema, la cantidad de células inflamatorias en la sangre, la producción de IgE en suero y los niveles de citocinas IL-1β, IL-6 y TNF-α en suero, pata y pulmones. Es importante mencionar, que el proceso de microencapsulación no mejoró de forma significativa el efecto antiinflamatorio comparado con el extracto sin microencapsular, por lo que no es necesario realizar este proceso.

Palabras clave: *Lippia graveolens*, edema de pata, inflamación de vías respiratorias, antiinflamatorio, modelo de ratones.

ABSTRACT

Inflammation is the process by which the immune system recognizes and eliminates harmful and foreign stimuli. During inflammation, some cells promote the production of proinflammatory mediators such as interleukin IL-1β, IL-6, IL-8, TNF-α, and ROS, among others. The overproduction of these mediators has been linked to the appearance of chronic degenerative diseases. For this purpose, steroidal and non-steroidal anti-inflammatory drugs are used. However, the use of these drugs has many side effects. As an alternative and adjuvant in the treatment of inflammation, the oregano plant has been used for decades, since it has been reported that it contains bioactive compounds (flavonoids) to which anti-inflammatory properties have been attributed. However, once extracted, flavonoids must be cost-effective, that is to be effective against diseases and have a long shelf life. Microencapsulation is used for this purpose. For all the above reasons, the aim of this project is to evaluate the anti-inflammatory potential of flavonoids in extract and oregano (*Lippia graveolens*) microcapsules on a mouse model with inflammation induced by carrageenan and ovalbumin. Extracts were obtained using different extraction methods (maceration, ultrasound-assisted extraction and supercritical fluid extraction). The content of total phenolic compounds, total flavonoids and antioxidant capacity using multiple methods (ABTS, DPPH, FRAP and ORAC) was evaluated. Flavonoids in each extract were also identified and quantified by UPLC-TQS-MS/MS, and the extract was microencapsulated. Paw edema in mice was induced with carrageenan, while they were sensitized and exposed to allergic asthma with ovalbumin. *L. graveolens* samples were administered orally to mice to assess edema size. Inflammatory cell count, serum IgE level and detection of proinflammatory cytokine levels in serum, plantar tissue and lung tissue were analyzed. Fourteen flavonoids were identified and quantified. Treatments with *L. graveolens* significantly reduced edema size, the number of inflammatory cells in the blood, serum IgE production and levels of cytokines IL-1β, IL-6 and TNF-α in serum, paw and lungs. It is important to mention that the microencapsulation process did not significantly improve the anti-inflammatory effect compared to the non-microencapsulated extract, so it is not necessary to carry out this process.

Keywords: *Lippia graveolens*, paw edema, airway inflammation, anti-inflammatory, mice models.

1. SINOPSIS

1.1. Justificación

En el mundo, tres de cada cinco personas mueren debido a enfermedades inflamatorias crónicas, entre estas destacan accidentes cerebrovasculares, trastornos cardiacos, enfermedades respiratorias crónicas, cáncer, obesidad y diabetes, entre otras. Los tratamientos utilizados son medicamentos antiinflamatorios no esteroideos y corticoesteroides, aunque estos presentan una diversidad de efectos secundarios en las personas. Muchos trabajos de investigación están enfocados en descubrir tratamientos alternativos o coadyuvantes para regular los procesos inflamatorios y reducir las enfermedades relacionadas a este proceso. Para ello, principalmente se utilizan compuestos naturales de plantas, los cuales tienen propiedades bioactivas. Entre estos compuestos destacan los flavonoides, los cuales han demostrado que participan en la regulación transcripcional de mediadores inflamatorios, en esta regulación se encuentra la inhibición de rutas de señalización que regulan la producción de citocinas y enzimas proinflamatorias, entre ellas la ruta del NF-κB y de las MAPKs. Por ello, estos compuestos reducen los niveles de inflamación y presentan un menor desarrollo de efectos secundarios en las personas. Sin embargo, para una mayor bioactividad, varias investigaciones proponen encapsular estos compuestos con el fin de protegerlos de diferentes factores ambientales y gastrointestinales, para evitar su degradación y controlar su liberación al ser consumidos.

En este sentido, los extractos libres y encapsulados de orégano (*L. graveolens*) podrían presentar un alto potencial antiinflamatorio debido a su alto contenido de flavonoides. Dicho esto, el orégano es una alternativa como coadyuvante o desarrollo de tratamientos contra la inflamación.

1.2. Antecedentes

1.2.1. Inflamación

La inflamación es la respuesta del sistema inmunitario a estímulos nocivos, como patógenos, células dañadas, compuestos tóxicos o irradiación, y actúa eliminando dichos estímulos e iniciando el proceso de curación. La inflamación es, por tanto, un mecanismo de defensa vital para la salud el cual puede ser agudo o crónico de acuerdo con el tipo de estímulo y o tejidos lesionados. La inflamación aguda puede ocurrir mediante el daño tisular provocado por traumatismos, invasión microbiana o compuestos nocivos; esté tipo de inflamación comienza rápidamente y los síntomas pueden durar unos días, por ejemplo, celulitis o neumonía aguda. A nivel tisular, la inflamación aguda se caracteriza por enrojecimiento, hinchazón, calor, dolor y pérdida de la función tisular, que resultan de las respuestas de las células inmunitarias, vasculares e inflamatorias locales a la infección o lesión. Por otro lado, la inflamación crónica también conocida como inflamación lenta a largo plazo, dura períodos prolongados de varios meses a años. En general, la extensión y los efectos de la inflamación crónica varían según la causa de la lesión y la capacidad del cuerpo para reparar y superar el daño (Chen *et al*., 2018; Pahwa *et al*., 2021).

Los eventos microcirculatorios importantes que ocurren durante el proceso inflamatorio incluyen cambios en la permeabilidad vascular, reclutamiento y acumulación de leucocitos y liberación de mediadores inflamatorios. En respuesta a la lesión del tejido, el cuerpo inicia una cascada de señales químicas que estimula las respuestas destinadas a curar los tejidos afectados (Chen *et al*., 2018).

1.2.2. Mecanismo de Respuesta Inflamatoria

La respuesta inflamatoria consiste en la activación coordinada de varias vías de señalización que regulan los niveles de mediadores inflamatorios en las células presentes en los tejidos y en las

células inflamatorias provenientes de la sangre. Aunque la respuesta inflamatoria varía según el tipo de estímulo inicial y su ubicación en el organismo, todos estos procesos comparten un mecanismo similar que se puede describir de la siguiente manera: 1) los receptores de la superficie celular identifican estímulos dañinos; 2) se activan las vías de inflamación; 3) se liberan los marcadores inflamatorios; y 4) se atraen células inflamatorias (Chen *et al*., 2018).

1.2.2.1. Activación del receptor de reconocimiento de patrones. Los receptores de reconocimiento de patrones (PRR, por sus siglas en inglés) se encuentran en compartimentos subcelulares como las membranas celulares y endosómicas, en el citosol, y también extracelularmente en formas secretadas que circulan en la sangre y los líquidos intersticiales (Amarantes-Mendes *et al*., 2018). En el sistema inmune innato de los vertebrados, la mayoría de los PRR pueden agruparse en cinco tipos según la similitud de sus dominios proteicos: 1) receptores tipo Toll (TLR), 2) receptores tipo NOD (NLR), 3) receptores inducidos por ácido retinoico I (RIG-I) (RLR), 4) receptores de lectina tipo C (CLR), y 5) receptores ausentes en melanoma-2 (AIM2) (ALR). Los PRR están compuestos básicamente por dominios para el reconocimiento de ligandos, dominios intermedios y dominios efectores (Li *et al*., 2021). Algunos de estos receptores se encuentran en la superficie de las células, permitiendo la vigilancia del entorno extracelular. Otros receptores como los NLR, RLR y algunos TL) se activan mediante estímulos inflamatorios que ingresan a la célula, como el ADN o ARN. La activación de estos PRR desencadena la producción de mediadores inflamatorios, que contribuyen a la eliminación de patógenos o al restablecimiento de la homeostasis del tejido. No obstante, una activación prolongada de estos receptores puede llevar al desarrollo de enfermedades inflamatorias (Chen *et al*., 2018**).**

1.2.2.2. Activación de vías inflamatorias. Las vías inflamatorias influyen en el desarrollo de varias enfermedades crónicas y participan en procesos que involucran mediadores inflamatorios y rutas reguladoras comunes. Los productos de estímulos inflamatorios iniciales, como productos de microorganismos y citocinas promueven la inflamación al interactuar con los receptores de reconocimiento. La activación de estos receptores desencadena importantes rutas de señalización intracelular, como las vías de la proteína quinasa activada por mitógenos (MAPK), el factor nuclear

kappa-B (NF-κB) y el sistema de transducción de señales y activación de la transcripción (STAT) de la quinasa Janus (JAK). A continuación, se describen cada una de estas vías.

1.2.2.2.1. Vía NF-κB. Un factor de transcripción nuclear es una proteína que se une a secuencias específicas de nucleótidos en la región promotora de un gen para iniciar su transcripción. Uno de los factores de transcripción nuclear más relevantes es el factor nuclear de la cadena ligera κ de células B activadas (NF-κB), una proteína con diversas funciones biológicas y presente en casi todas las células de mamíferos (Zhang *et al*., 2021). NF-κB es conocido por su papel en la regulación de respuestas inflamatorias, actuando como un activador clave de varios genes proinflamatorios. Además de promover la expresión de múltiples genes proinflamatorios en células inmunitarias innatas, NF-κB regula la activación, diferenciación y función de las células T inflamatorias.

En mamíferos, NF-κB es en realidad una familia de cinco proteínas que pueden formar dímeros para influir positiva o negativamente en la transcripción de genes. Las cinco subunidades son NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB y c-Rel, las cuales regulan la transcripción de genes específicos al unirse a un elemento de ADN denominado potenciador de κB, ya sea como heterodímeros o homodímeros (Mitchell *et al*., 2018). La activación de NF-κB sigue dos vías de señalización principales: la vía canónica y la no canónica (o alternativa), ambas importantes en la regulación de respuestas inmunitarias e inflamatorias, aunque con mecanismos de señalización distintos. La vía canónica responde a diferentes estímulos, como ligandos de receptores de citocinas, receptores de reconocimiento de patrones (PRR), miembros de la superfamilia de receptores TNF (TNFR), y también receptores de células T (TCR) y células B. Esta vía participa en casi todos los aspectos de la respuesta inmunitaria, mientras que la vía no canónica parece funcionar como una señalización complementaria, colaborando con la vía canónica en la regulación de funciones específicas del sistema inmunitario adaptativo (Liu *et al*., 2017). Debido a la gran variedad de factores inflamatorios, que incluyen estímulos tanto infecciosos como no infecciosos, el papel de NF-κB en la inflamación es complejo.

1.2.2.2.2. Vía MAPK. Las MAPK son una familia de quinasas de serina/treonina que regulan las respuestas celulares ante diversos estímulos, tales como el estrés osmótico, mitógenos, choque

térmico y citocinas inflamatorias (como IL-1, TNF-α e IL-6), desempeñando un papel clave en la proliferación, diferenciación, supervivencia celular y apoptosis. En los mamíferos, las MAPK incluyen la cinasa ERK1/2 regulada por señales extracelulares, la cinasa p38 MAP y las c-Jun N-terminal cinasas (JNK). Cada vía de señalización de MAPK involucra al menos tres componentes: una MAPK, una MAPK quinasa (MAPKK) y una MAPK quinasa quinasa (MAPKKK). Generalmente, los mitógenos y las señales de diferenciación activan las ERK, mientras que los estímulos inflamatorios y el estrés activan JNK y p38. La activación de las MAPK, como Erk1/2 y JNK, provoca la fosforilación y activación de los factores de transcripción p38v en el citoplasma o núcleo, lo que desencadena la respuesta inflamatoria (Sun *et al*., 2016).

1.2.2.2.3. Vía JAK-STAT. Las proteínas transductoras de señales y activadoras de la transcripción (STAT) son algunos de los factores de transcripción más conservados y poderosos. Existen siete proteínas STAT (STAT1, 2, 3, 4, 5a, 5b y 6), que se identificaron inicialmente como factores de transcripción latentes en el citoplasma, activados por fosforilación de tirosina en respuesta a la estimulación de citocinas y factores de crecimiento. La unión de un factor de crecimiento o citocina a su receptor correspondiente provoca un cambio conformacional y la activación de la quinasa Janus asociada al receptor (JAK1, JAK2, JAK3 o Tyk2), lo que les permite autofosforilarse y fosforilar la cola citoplasmática del receptor en residuos de tirosina (Gurzon *et al*., 2016). La vía JAK-STAT, muy conservada, involucra diversas citocinas, factores de crecimiento, interferones y otras moléculas, como la leptina y la hormona del crecimiento. Este mecanismo de señalización permite que los factores extracelulares controlen la expresión génica. Las JAK asociadas a receptores se activan mediante la unión de ligandos y se fosforilan entre sí, creando sitios de unión para los STAT, que son factores de transcripción citoplasmáticos latentes. Los STAT reclutados a estos sitios sufren fosforilación y dimerización antes de trasladarse al núcleo. La fosforilación de tirosina es crucial para la dimerización de STAT y su unión al ADN, lo que permite que la señalización JAK/STAT traduzca directamente una señal extracelular en una respuesta transcripcional. Un ejemplo de esto es la unión de los miembros de la familia IL-6 a los receptores de la membrana plasmática, lo que activa las proteínas JAK-STAT. Las proteínas STAT que se trasladan al núcleo se unen a las regiones promotoras de los genes diana, regulando la transcripción de genes inflamatorios (Chen *et al*., 2018).

La desregulación de las actividades de NF-κB, MAPK o JAK-STAT está asociada con enfermedades inflamatorias, autoinmunes, metabólicas y cáncer. La señalización a través de factores de transcripción resulta en la secreción de citocinas. Diversos factores de transcripción regulan una variedad de genes inflamatorios, como IL-1, TNF-α, IL-6, factor estimulante de colonias (CSF), interferones, factor de crecimiento transformante (TGF) y quimiocinas.

1.2.2.3. Marcadores inflamatorios. Los marcadores se utilizan en el ámbito clínico para identificar procesos biológicos normales frente a patológicos y para evaluar las respuestas a tratamientos terapéuticos. Los marcadores inflamatorios pueden ser indicadores de enfermedades inflamatorias, y están relacionados con las causas y consecuencias de diversas patologías inflamatorias, como enfermedades cardiovasculares, disfunciones endoteliales e infecciones. Los estímulos activan células inflamatorias, como los macrófagos y adipocitos, lo que induce la producción de citocinas inflamatorias, como IL-1β, IL-6, TNF-α, así como proteínas y enzimas inflamatorias. Estas moléculas podrían ser utilizadas como biomarcadores para el diagnóstico de enfermedades, para predecir su evolución y para apoyar la toma de decisiones terapéuticas.

1.2.2.3.1. Citocinas inflamatorias. Las citocinas son liberadas principalmente por las células inmunitarias, como monocitos, macrófagos y linfocitos. Las citocinas proinflamatorias y antiinflamatorias desempeñan roles opuestos, facilitando o inhibiendo la inflamación, respectivamente. Las citocinas inflamatorias incluyen IL, factores estimulantes de colonias (CSF), interferones (IFN), TNF, TGF y quimiocinas, son producidas por las células principalmente para reclutar leucocitos al sitio de infección o lesión. Estas citocinas modulan la respuesta inmunitaria frente a infecciones o inflamación, y regulan la inflamación misma a través de una compleja red de interacciones. Sin embargo, una producción excesiva de citocinas inflamatorias puede causar daño en los tejidos, alteraciones hemodinámicas, insuficiencia orgánica e incluso la muerte. Las citocinas más conocidas por estimular y perpetuar las respuestas inflamatorias son IL-1β, IL-6 y TNF-α (Chen *et al*., 2018).

a) **IL-6.** Esta fue identificada originalmente como un factor de diferenciación de células B, y se ha asociado con niveles elevados de esta citoquina, que activan células B policlonales y provocan

inflamación crónica. En las fases tempranas de la inflamación aguda, la IL-6 media la respuesta de fase aguda. Durante procesos inflamatorios crónicos, los niveles elevados de IL-6 favorecen la supervivencia y el crecimiento de linfocitos y macrófagos, lo que perpetúa la inflamación.

b) **IL-1β.** Esta presenta diversas actividades directas e indirectas que favorecen la inflamación, como estimular la producción de otras citocinas y la liberación de prostaglandinas. Estas acciones contribuyen a la generación de células efectoras citotóxicas y actúan sinérgicamente con factores estimulantes de colonias para incrementar la producción de células inflamatorias en la médula ósea.

c) **TNF-α.** Esta incrementa la inflamación y juega un papel clave en la eliminación de células muertas y moribundas a través de la apoptosis. Se ha demostrado que TNF-α regula positivamente la expresión de moléculas del complejo principal de histocompatibilidad (MHC) de clase I y II en ciertos tipos celulares, lo que resulta en la activación celular y la liberación de citocinas (Germolec *et al*., 2018). Una mejor comprensión de cómo regular las vías de las citocinas podría permitir una identificación más precisa de la inflamación mediada por agentes y mejorar el tratamiento de enfermedades inflamatorias.

1.2.2.3.2. Proteínas y enzimas inflamatorias. Las proteínas inflamatorias presentes en la sangre, como la proteína C reactiva (PCR), la haptoglobina, el amiloide A sérico, el fibrinógeno y la glicoproteína ácida alfa 1, desempeñan un papel crucial en la restauración de la homeostasis y en la inhibición del crecimiento microbiano durante episodios de traumatismo, estrés o infección, independientemente de los anticuerpos. La activación anómala de ciertas enzimas, como la caja 1 del grupo de alta movilidad (HMGB1), superóxido dismutasa (SOD), glutatión peroxidasa (GPx), NADPH oxidasa (NOX), óxido nítrico sintasa inducible (iNOS) y ciclooxigenasa (COX)-2, tiene un papel fundamental en el desarrollo de enfermedades inflamatorias, como las enfermedades cardiovasculares y el cáncer. Por ejemplo, los efectos extracelulares de HMGB1 pueden ser mediados por la activación de vías de señalización asociadas a los receptores TLR. El principal objetivo extracelular de HMGB1 es TLR4, lo que activa cascadas de señalización intracelular dependientes de MyD88, las cuales están involucradas en la activación de las vías NF-κB y MAPK. Esto da lugar a la liberación de citocinas inflamatorias como TNF-α e IL-1β. Las proteínas y enzimas inflamatorias se han utilizado como biomarcadores para la inflamación, infección y trauma en medicina (Chen *et al*., 2018; Germolec *et al*., 2018).

1.2.2.4. Tipos de células en las respuestas inflamatorias. La respuesta inflamatoria implica una red altamente coordinada de diferentes tipos de células. Los macrófagos, monocitos y otras células activadas median las respuestas locales ante el daño tisular y la infección. En los sitios de lesión, las células epiteliales y endoteliales dañadas liberan factores que inician la cascada inflamatoria, junto con quimiocinas y factores de crecimiento, que atraen a neutrófilos y monocitos. Las primeras células en llegar al sitio de la lesión son los neutrófilos, seguidos por los monocitos, linfocitos, células asesinas naturales (células NK), células T, células B y mastocitos. Los monocitos pueden diferenciarse en macrófagos y células dendríticas, y son reclutados a los tejidos dañados mediante quimiotaxis. Las alteraciones en las células inmunitarias mediadas por la inflamación están asociadas con varias enfermedades, como asma, cáncer, enfermedades inflamatorias crónicas, aterosclerosis, diabetes, así como enfermedades autoinmunes y degenerativas. Los neutrófilos, que combaten microorganismos, también pueden causar daño en las células y tejidos del huésped. Son mediadores clave de la respuesta inflamatoria y ayudan a activar las células T mediante la programación de las células presentadoras de antígenos, además de liberar factores locales que atraen a monocitos y células dendríticas. Los macrófagos son componentes esenciales del sistema de fagocitos mononucleares y desempeñan un papel crítico en el inicio, mantenimiento y resolución de la inflamación. Durante este proceso, los macrófagos presentan antígenos, realizan fagocitosis y modulan la respuesta inmunitaria a través de la producción de citocinas y factores de crecimiento. Los mastocitos, ubicados en las matrices del tejido conjuntivo y superficies epiteliales, son células efectoras que inician respuestas inflamatorias. Cuando se activan, liberan una variedad de mediadores inflamatorios, como citocinas, quimiocinas, histamina, proteasas, prostaglandinas, leucotrienos y serglicina proteoglicanos. Varios estudios han demostrado que las plaquetas influyen en los procesos inflamatorios, desde la aterosclerosis hasta las infecciones. Las interacciones entre las plaquetas y las células inflamatorias pueden mediar resultados proinflamatorios. La respuesta de fase aguda (APR) es la respuesta temprana ante la infección o lesión, y algunos estudios sugieren que las plaquetas inducen esta respuesta. Tras ser reclutadas por estímulos inflamatorios, las células inmunitarias amplifican y mantienen la APR mediante la liberación de mediadores inflamatorios locales en el sitio de reclutamiento (Chen *et al*., 2018; Germolec *et al*., 2018**).**

1.2.3. Tratamientos

Algunos fármacos convencionales que combaten la inflamación crónica son: medicamentos antiinflamatorios no esteroideos y corticosteroides. Los medicamentos antiinflamatorios no esteroideos (AINE) como el naproxeno, el ibuprofeno y la aspirina actúan inhibiendo una enzima ciclooxigenasa (COX) que contribuye a la inflamación y se usan principalmente para aliviar el dolor causado por la inflamación en pacientes con artritis. Los corticosteroides también previenen varios mecanismos involucrados en la inflamación. Los glucocorticoides se utilizan para diferentes afecciones inflamatorias, como la artritis inflamatoria, el lupus sistémico, la sarcoidosis y el asma (Pahwa *et al*., 2022). Sin embargo, todos estos medicamentos tienen la característica de presentar efectos secundarios mismos que provocan problemas de salud que pueden ser de leves a graves. Por ello, se buscan alternativas complementarias que puedan ser más efectivas y seguras contra la inflamación, como podrían ser los compuestos vegetales naturales.

1.2.4. Orégano y generalidades

El orégano mexicano (*L. graveolens*) es una planta aromática originaria de México y América Central. Aunque se le conoce como orégano, pertenece a una familia botánica distinta del orégano mediterráneo (*O. vulgare*), mostrando características únicas tanto en sabor como en propiedades medicinales. Es un arbusto perenne que alcanza entre 1 y 3 m de altura, el cual presenta hojas verdes, pequeñas, ovaladas y opuestas con bordes ligeramente dentados; ademas, sus flores son pequeñas, de color blanco o amarillento reunidas en inflorescencias terminales. Esta planta es ideal para climas secos y áridos ya que se adapta a altas temperaturas y suelos pobres (Rojas-Arechiga *et al*., 2014).

Los principales usos del orégano son en el ambito culinario y medicinal. Este es un condimento esencial en la cocina mexicana. Es popular en la preparación de platillos típicos de México como el pozole y tacos al pastor entre una gran diversidad. Su sabor es más cítrico y robusto que el orégano europeo, lo que lo hace único en la gastronomía. Por otro lado, el orégano mexicano tiene

usos en la medicina tradicional como antimicrobiano, antifúngico, antiinflamatorio, digestivo, antioxidante y expectorante. Así como también sus aceites esenciales son utilizados en las industrias farmacéuticas, cosméticas y de alimentos (Soto *et al*., 2018).

El orégano mexicano es una especie de interés comercial, tanto en el mercado nacional como internacional. México es uno de los principales exportadores de orégano debido a su alta calidad y aroma característico. Durante el 2019, se produjeron 147 ton, siendo Estado de México, San Luis Potosí, Baja California y Oaxaca, los principales estados productores (SIAP, 2021).

El orégano mexicano es una planta con un alto valor culinario, medicinal e industrial. Su adaptabilidad a climas áridos y su potente perfil químico lo convierten en un recurso importante para la gastronomía y la industria de productos naturales. Sin embargo, las investigaciones se dirigen al estudio de sus propiedades funcionales y nutracéuticas destacando su propiedad antiinflamatoria, las cuales son atribuidas a los compuestos fenólicos que contiene, principalmente flavonoides.

1.2.5. Flavonoides

Los flavonoides son compuestos secundarios que se caracterizan principalmente por un anillo de benzopirona con grupos fenólicos o polifenólicos en diversas posiciones. Se encuentran principalmente en frutas, hierbas, tallos, cereales, nueces, vegetales, flores y semillas. Los componentes fitoquímicos bioactivos presentes en estas partes de las plantas les confieren propiedades medicinales y biológicas. Hasta la fecha, se han aislado e identificado más de 10,000 flavonoides, muchos de los cuales son reconocidos como agentes terapéuticos, por ejemplo quercetina y naringenina como potentes antioxidantes y antiinflamatorios. Su síntesis ocurre naturalmente a través de la ruta de los fenilpropanoides, con una bioactividad que depende de su absorción y biodisponibilidad (Ullah *et al*., 2020).

Los flavonoides se pueden clasificar en diferentes grupos según el carbono del anillo C al que se une el anillo B, así como el grado de insaturación y oxidación del anillo C. Los flavonoides en los que el anillo B se conecta en la posición 3 del anillo C se conocen como isoflavonas. Aquellos con el anillo B en la posición 4 se llaman neoflavonoides, y los que lo tienen en la posición 2 se dividen

en varios subgrupos, dependiendo de las características estructurales del anillo C. Estos subgrupos incluyen flavonas, flavonoles, flavanonas, flavanonoles, flavanoles o catequinas, antocianinas y chalconas (Panche *et al*., 2016).

1.2.6. Métodos de extracción de flavonoides de orégano

Existe un enfoque general que consta de tres etapas para aislar, extraer e identificar fitoquímicos de fuentes naturales. La primera etapa, el pretratamiento o preparación de la muestra, incluye procesos como centrifugado, filtración y secado, entre otros. La segunda etapa se centra en la extracción, aislamiento y purificación de los flavonoides de diversas muestras vegetales, utilizando métodos como la extracción con Soxhlet, maceración, infusión en agua, fluidos supercríticos, extracción en microfase sólida, microondas, ultrasonido, autohidrólisis, etc. Finalmente, en la tercera etapa, los extractos purificados y extraídos se analizan mediante técnicas cromatográficas, que permiten la identificación, cuantificación y recuperación de los compuestos flavonoides. Los métodos de extracción se dividen en convencionales y emergentes, y son aplicados en investigación para la extracción de flavonoides.

1.2.6.1. Métodos convencionales. La extracción y recuperación de flavonoides ha ganado relevancia en los últimos años debido a la creciente preferencia de la población por estilos de vida más saludables y la incorporación de antioxidantes en la dieta. Como resultado, se han desarrollado diversos métodos para extraer flavonoides con el fin de mejorar los rendimientos de estos importantes compuestos bioactivos. Entre los métodos propuestos se encuentran la maceración, percolación, hidrodestilación, ebullición, reflujo, remojo y Soxhlet. Se han probado solventes como etanol, metanol, benceno, cloroformo y acetato de etilo, entre otros, para evaluar su efecto sobre los rendimientos de extracción (Chávez-González *et al*., 2020).

En general, la extracción líquido-líquido o sólido-líquido es la técnica más utilizada para obtener flavonoides. Aunque la maceración y la infusión de agua son métodos tradicionales, todavía se emplean hoy en día. Estos procesos han incorporado solventes como etanol, metanol y acetona,

además de agua, para extraer compuestos bioactivos. Los métodos tradicionales de extracción se caracterizan por la utilización de grandes cantidades de solvente, menores rendimientos y tiempos de extracción más largos en comparación con otros enfoques. Se ha observado que cuando los métodos de extracción incluyen tratamientos térmicos, las estructuras químicas de los flavonoides extraídos pueden degradarse, lo que reduce su bioactividad. Factores como el tiempo, el tamaño de partícula, el tipo de solvente, la relación masa/volumen y la temperatura han sido evaluados en los métodos tradicionales de extracción de flavonoides. El tipo de solvente utilizado influye directamente en el tipo de flavonoide extraído y en la actividad biológica de los compuestos obtenidos; de los solventes probados, el etanol y el metanol son los más empleados debido a que ofrecen mayores rendimientos en la recuperación de flavonoides (Chávez-González *et al*., 2020).

1.2.6.2. Métodos emergentes. Actualmente se siguen desarrollando continuamente técnicas avanzadas para superar las limitaciones de los métodos tradicionales en la extracción de flavonoides. Entre estas técnicas se incluyen la extracción asistida por ultrasonido (EAU), la extracción con fluido supercrítico (EFS), la extracción asistida por microondas (EAM), la extracción en fase sólida (EFS), la extracción asistida por enzimas, la extracción con líquido presurizado (PLE), la extracción acelerada con solventes (EAS), la extracción asistida por campo eléctrico pulsado (EACE) y combinaciones de estas metodologías. Estas técnicas modernas son muy eficaces para extraer flavonoides de una amplia variedad de matrices naturales. Lo más destacado es que también permiten reducir el uso de solventes orgánicos, sustituyéndolos por solventes alternativos "verdes", todo ello mientras logran altos rendimientos en tiempos de extracción más cortos. Por esta razón, estas metodologías se consideran parte de las técnicas de “extracción verde” (Chaves *et al*., 2020).

Dado que las matrices de las muestras y las características químicas de los flavonoides son complejas, los expertos coinciden en que no existe un método único y estándar para extraer estos compuestos de todos los materiales o flavonoides en la actualidad.

1.2.7. Mecanismos de acción antiinflamatorio de flavonoides de orégano

Los mecanismos antiinflamatorios reportados para flavonoides son la regulación transcripcional de mediadores inflamatorios, en esta regulación se encuentra la inhibición de rutas de señalización que regulan la producción de citocinas y enzimas proinflamatorias, entre ellas la ruta del NF-κB y de las MAPKs. El mecanismo de los flavonoides puede estar relacionado con la inhibición de la vía factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-κB) inducido por el factor de necrosis tumoral-α (TNF-α) para suprimir la ciclooxigenasa-2 (COX-2) y expresiones de óxido nítrico sintasa inducible (iNOS). En general, se ha sugerido que los mecanismos moleculares involucrados en las actividades antiinflamatorias de los flavonoides incluyen: inhibición de enzimas proinflamatorias, como COX2, lipoxigenasa y iNOS, inhibición de NF-kB y proteína activadora-1 (AP-1) y la activación de las enzimas desintoxicantes antioxidantes de fase II, la proteína quinasa activada por mitógeno (MAPK), la proteína quinasa C y el factor 2 relacionado con el factor eritroide 2 nuclear (Li *et al*., 2020; Zhazykbayeva *et al*., 2020; Li *et al*., 2022).

Los compuestos bioactivos del orégano como lo son los flavonoides, se han destacado por su alto potencial antioxidante y antiinflamatorio. Algunas investigaciones que han dado pauta a atribuirle estas propiedades son las siguientes: Leyva-López *et al*. (2016), evaluó extractos metanólicos de orégano *L. graveolens* rico en flavonoides como quercetina, escutelarina y floridzina, esto, en líneas celulares de macrófagos RAW 264.7 estimuladas con LPS, encontrando que estos compuestos tenían un efecto inhibitorio significativo sobre mediadores pro-inflamatorios (óxido nítrico y EROs).

También, Vucij *et al*. (2015) evaluaron los flavonoides naringenina y eriodictiol extraídos de orégano *O. vulgare*, en células aisladas de ratones C57BL/6 macho, encontrando que dichos compuestos reducían la expresión de citocinas pro-inflamatorias IL-17 murina, IL-1β, factor de necrosis tumoral (TNF), así como el aumento de citocinas antinflamatorias como IL-10, IFN-γ, IL-4.

En un estudio *in vivo*, aislaron de un extracto de orégano, los flavonoides naringenina e hidroxifloridzina, y los evaluaron en ratones CD1 macho con edema de oído inducido por 12-O-tetradecanoilforbol-13-acetato (TPA), destacando que redujeron la inflamación del edema en un

41.96% naringenina y 42.63% hidrozifloridzina (Amador *et al*., 2020). Por otra parte, en un estudio realizado por Chuang y cols en 2018, evaluaron un extracto etanólico de orégano *O. vulgare* rico en apigenina y quercetina, en ratones macho de ocho semanas con edema de oído inducido por *Propionibacterium acnes,* encontrando que los compuestos suprimían significativamente la inflamación de la piel inducida por *P. acnes*, medida por el grosor de la oreja (32%) y el peso de la biopsia (37%). En un estudio separado, utilizando el cocultivo de *P. acnes* y monocitos THP-1 humanos, el extracto de orégano redujo la producción de interleucina (IL)-8, IL-1β y factor de necrosis tumoral (TNF)-α hasta en un 40%, 37% y 18%. El compuesto más representativo de orégano: naringenina, ha demostrado poseer actividad antiinflamatoria y antioxidante en modelos *in vivo*, al ser aplicada en ratones con inflamación inducida por zymosan, logrando mejorar su estado de estrés oxidativo mediante aumento en los niveles y expresión del Nrf2, aumentando los niveles de GSH, y reduciendo la producción de anión superóxido (Bussman *et al*., 2019). Este mismo flavonoide, en un estudio de Zhao *et al*. (2018) inhibió la expresión y los niveles de la MAPKs p38, en un modelo de macrófagos murinos estimulados con lipopolisacáridos.

1.2.8. Referencias

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1.3 Hipótesis

1. ¿Qué efecto presentan diferentes métodos de extracción sobre el contenido de compuestos fenólicos totales, flavonoides totales, capacidad antioxidante y perfil cromatográfico de flavonoides de orégano (*L. graveolens*)?

H: La extracción ultrasonido-DES incrementa el contenido de compuestos fenólicos, flavonoides totales y la capacidad antioxidante; mientras que la extracción maceración- metanol incrementa el perfil y el contenido de flavonoides, destacando naringenina y floridzina.

2. ¿Cuál es el contenido de compuestos fenólicos totales, flavonoides totales, capacidad antioxidante y perfil cromatográfico de flavonoides de los extractos de orégano (*L. graveolens*) microencapsulados?

H: Las microcápsulas de orégano presentan un contenido de compuestos fenólicos y flavonoides totales mayor a 15 mg EAG/g y 5mg EQ/g de muestra, respectivamente. Así como una capacidad antioxidante entre los 150-700 μmoles ET/g de microcápsulas. Destacando los

flavonoides naringenina, floridzina, quercetina y luteolina-7-glucósido.

3. ¿Cuál es el efecto antiinflamatorio que presenta el extracto y microcápsulas de orégano (*L. graveolens*) sobre inflamación aguda inducida con carragenina en ratones?

H: El extracto y microcápsulas de orégano reducen significativamente la inflamación de la pata en ratones, además disminuye los niveles de células inflamatorias y reduce la expresión de marcadores proinflamatorios (IL-1β, IL-6 y TNF-α) en suero y tejido plantar.

4. ¿Cuál es el efecto antiinflamatorio del extracto y microcápsulas de orégano (*L. graveolens*) sobre la inflamación de vías respiratorias inducida con ovoalbúmina en ratones?

H: El extracto y microcápsulas de orégano reducen significativamente los niveles de células inflamatorias, la expresión de IgE y disminuye el contenido de citocinas proinflamatorias (IL-1β, IL-6 y TNF-α) en tejido pulmonar.

1.4. Objetivo General

Comparar diferentes métodos de extracción de flavonoides y evaluar la respuesta antiinflamatoria de los extractos y microcápsulas de orégano (*L. graveolens*) sobre inflamación inducida por carragenina y ovoalbúmina en modelos murinos.

1.5. Objetivos Específicos

1. Comparar el contenido de compuestos fenólicos totales, flavonoides totales, capacidad antioxidante (ABTS, FRAP, DPPH Y ORAC) y perfil cromatográfico de flavonoides (UPLC-TQS-MS/MS) en extractos de orégano (*Lippia graveolens*) obtenidos por maceración (agua y metanol), extracción asistida con ultrasonido (DES: etilenglicol, glicerol y ácido láctico) y extracción por fluido supercrítico (CO_2).
2. Determinar el contenido de compuestos fenólicos totales, flavonoides totales, capacidad antioxidante (ABTS, FRAP y ORAC) y perfil cromatográfico de flavonoides (UPLC-TQS-

MS/MS) en microcápsulas de orégano (*Lippia graveolens*).

3. Evaluar la actividad anti-inflamatoria de extracto y microcápsulas de orégano ricos en flavonoides, sobre ratones con edema de pata inducido por carragenina, midiendo el grosor de la pata, células inflamatorias y citocinas proinflamatorias (IL-1β, IL-6 y TNF-α) en suero y tejido plantar.
4. Evaluar la actividad anti-inflamatoria de los extractos y microcápsulas de orégano ricos en flavonoides, sobre ratones con inflamación de vías respiratorias inducida con ovoalbúmina, midiendo los niveles de células inflamatorias, niveles de IgE y la expresión de citocinas (IL-1β, IL-6 y TNF-α) en suero y tejido pulmonar.

1.6. Sección Integradora del Trabajo

La información presente en este documento esta dividida en secciones denominadas capítulos. A continuación, se describe el contenido de cada capítulo.

"Green extracts and UPLC-TQS-MS/MS profiling of flavonoids from Mexican oregano (*Lippia graveolens*) using natural deep eutectic solvents/ultrasound-assisted and supercritical fluids". Describe el trabajo sobre la comparación del contenido de compuestos fenólicos totales, flavonoides totales, capacidad antioxidante (ABTS, FRAP, DPPH Y ORAC) y perfil cromatográfico de flavonoides (UPLC-TQS-MS/MS) en extractos de orégano mexicano obtenidos por diferentes métodos de extracción utilizando tecnologías emergentes y convencionales tales como maceración con metanol y agua, extracción asistida por ultrasonido (UAE) utilizando solventes eutécticos profundos (DES) como cloruro de colina-etilenglicol, cloruro de colina-glicerol y cloruro de colina-ácido láctico, así como también la extracción con fluidos supercríticos usando CO_2. Los resultados mostraron que UAE-DES tuvo el mejor efecto de extracción y capacidad antioxidante utilizando métodos colorimétricos. Sin embargo, la maceración-metanol fue superior en contenido de compuestos, destacando la naringenina y la floridzina como los compuestos mayoritarios. Además, este extracto fue microencapsulado por secado por aspersión, lo que proporcionó una característica de protección de su potencial antioxidante. Los extractos y

microcápsulas de orégano ricos en flavonoides presentan resultados prometedores para futuras investigaciones. En este artículo se abordó la información relacionada a los objetivos específicos 1 y 2 de esta investigación.

"Anti-inflammatory effect of *Lippia graveolens* extracts and microcapsules in a model of carrageenan–induced paw edema in mice". Tuvo como objetivo evaluar los efectos del extracto y microcápsulas de orégano mexicano (*Lippia graveolens*) contra la inflamación aguda en un modelo de edema de pata inducido por carragenina en ratones. Además, se evaluó el efecto contra la inflamación crónica de las vías respiratorias de ratones con asma alérgica inducida por ovoalbúmina. Además, se registró el grosor de la pata y al final del estudio, se extrajo sangre y se determinó el estado de inflamación mediante técnicas bioquímicas (hematología) y moleculares (determinación de IgE total y citocinas IL-1β, IL-6 y TNF-α). Los resultados del estudio sugieren que *Lippia graveolens* mejora significativamente el proceso antiinflamatorio en la pata y las vías respiratorias, regula los niveles de células inflamatorias en sangre y los niveles de inmunoglobulina E en suero sanguíneo. También ayuda a la reducción de citocinas proinflamatorias como IL-1β, IL-6 y TNF-α. Con estos resultados se confirmó la importancia farmacológica de *Lippia graveolens* frente a la inflamación aguda y crónica. En este artículo se abordó la información relacionada a los objetivos específicos 3 y 4 de esta investigación.

"Conclusiones generales". Se describen las conclusiones de acuerdo con los objetivos planteados en este trabajo.

"Recomendaciones". En esta sección se plantean las recomendaciones para la continuación de futuras investigaciones relacionadas con nuestro tema de estudio.

2. GREEN EXTRACTS AND UPLC-TQS-MS/MS PROFILING OF FLAVONOIDS FROM MEXICAN OREGANO (*Lippia graveolens*) USING NATURAL DEEP EUTECTIC SOLVENTS/ULTRASOUND-ASSISTED AND SUPERCRITICAL FLUIDS

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Revista:

Plants

Fecha de publicación:

18 de abril del 2023

DOI:

https://doi.org/10.3390/plants12081692

plants

MDPI

Article

Green Extracts and UPLC-TQS-MS/MS Profiling of Flavonoids from Mexican Oregano (*Lippia graveolens*) Using Natural Deep Eutectic Solvents/Ultrasound-Assisted and Supercritical Fluids

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Abstract: Mexican oregano (*Lippia graveolens*) is an important source of bioactive compounds, such as flavonoids. These have presented different therapeutic properties, including antioxidant and anti-inflammatory; however, their functionality is related to the quantity and type of compounds, and these characteristics depend on the extraction method used. This study aimed to compare different extraction procedures to identify and quantify flavonoids from oregano (*Lippia graveolens*). Emerging and conventional technologies include maceration with methanol and water, and ultrasound-assisted extraction (UAE) using deep eutectic solvents (DES) such as choline chloride-ethylene glycol, choline chloride-glycerol, and choline chloride-lactic acid. Supercritical fluid extraction using CO_2 as a solvent was also studied. Six different extracts were obtained and the total reducing capacity, total flavonoid content, and antioxidant capacity by ABTS$^{\bullet+}$, DPPH$^{\bullet}$, FRAP, and ORAC were evaluated. In addition, flavonoids were identified and quantified by UPLC-TQS-MS/MS. Results showed that UAE-DES had the best extraction effect and antioxidant capacity using colorimetric methods. However, maceration-methanol was superior in compound content, and highlighting naringenin and phloridzin were the major compounds. In addition, this extract was microencapsulated by spray drying, which provided a protection feature of their antioxidant potential. Oregano extracts are rich in flavonoids and the microcapsules present promising results for future research.

Keywords: flavonoids; oregano; antioxidant capacity; extraction techniques; microcapsules; chromatographic profile

Citation: Bernal-Millán, M.d.J.; Carrasco-Portugal, M.d.C.; Heredia, J.B.; Bastidas-Bastidas, P.d.J.; Gutiérrez-Grijalva, E.P.; León-Félix, J.; Angulo-Escalante, M.Á. Green Extracts and UPLC-TQS-MS/MS Profiling of Flavonoids from Mexican Oregano (*Lippia graveolens*) Using Natural Deep Eutectic Solvents/Ultrasound-Assisted and Supercritical Fluids. *Plants* **2023**, *12*, 1692. https://doi.org/10.3390/plants12081692

Academic Editor: Ivan Kreft

Received: 20 March 2023
Revised: 14 April 2023
Accepted: 16 April 2023
Published: 18 April 2023

1. Introduction

Mexican oregano (*Lippia graveolens*) is a plant that has taken importance in the scientific field for its high phytochemical content. Among these compounds, flavonoids stand out as hydrophilic compounds produced by plants to defend against biotic and abiotic factors [1]. Pharmacologically, these compounds are known for their low toxicity and antioxidant, antihypertensive, anti-inflammatory, antiproliferative, and antiviral properties [2]. Among the main flavonoids reported in oregano are quercetin, naringenin, and pinocembrin [3–5]. Their functionality is related to the compound type (structure) and concentration. However, these characteristics depend on the extraction method used; there are conventional and unconventional methods [6]. The traditional methods are simple to execute and lead to high extraction yields; however, they are not selective. The methodology developers have opted for using solvents such as ethanol, methanol, and acetone, and not just water to extract bioactive compounds. Parameters such as time, particle size, type of solvent,

mass/volume relation, and temperature, among others, have been evaluated in conventional flavonoid extraction methods [7]. On the other hand, unconventional methods, also known as "green extraction methods" or emerging technologies, are new and promising techniques to overcome the limitations of classic extraction methods. Within these methods are ultrasound-assisted extraction using deep eutectic solvents (DES) and supercritical fluid extraction using CO_2 as a solvent. The advantages they provide are reduced sample degradation and organic solvent consumption, contamination prevention, improved extraction efficiency, selectivity, and automation capability. [8]. The extraction of bioactive compounds from rosemary, clove, green tea, and oregano by different environmentally friendly techniques has been reported. Nonetheless, little has been studied about the use of DES on polyphenols and even less on this genus of plants [9]. However, regardless of the extraction method, during the handling and extraction of these compounds, their stability, efficacy, and functionality against diseases caused by pathogens or free radicals may be compromised, affecting feasibility and profitability [10]. For this reason, microencapsulation is the technology used to protect bioactive compounds from degradation or oxidation, reduce incompatibility problems, and control the release of the encapsulated active compound [11]. This study aimed to compare different extraction procedures for identifying and quantifying oregano (*Lippia graveolens*) flavonoids using various conditions, including solvent extraction with conventional and emerging technologies. Additionally, the extracts were analyzed by the UPLC-TQS-MS/MS, and their antioxidant potential was measured using in vitro methods. Finally, the extract with the best chromatographic profile of flavonoids was microencapsulated and its antioxidant characterization was carried out.

2. Results and Discussion

2.1. Total Phenolic and Flavonoid Content

The total phenolic compounds content was higher in the ultrasound-assisted extraction using deep eutectic solvents (DES) (Table 1). The results ranged between 17.5 and 126.1 mg GAE/g, with the choline chloride-lactic acid extract showing the highest concentration. However, no significant differences were found between the choline chloride-glycerol and choline chloride-ethylene glycol extracts. The methanolic extract also obtained significant concentrations of total phenolics with 66.2 mg GAE/g, while the water and supercritical CO_2 extracts had the lowest content with 27.9 and 17.5 mg GAE/g, respectively. The higher extraction capacity of flavonoids with DES can be attributed to the H-bond interactions between the phenolic compound molecules and the plant cell wall structure alteration by acoustic cavitation [12,13].

Table 1. Total phenolic and total flavonoid contents in oregano extracts (*Lippia graveolens*).

Sample/Extracts	Total Phenolic (mg GAE/g)	Total Flavonoids (mg QE/g)
Methanol	66.2 ± 3.4^{b}	26.9 ± 1.8^{bc}
Water	27.9 ± 3.4^{c}	19.2 ± 0.3^{c}
ChCl-Et	100.1 ± 7.5^{ab}	39.5 ± 3.3^{a}
ChCl-Gly	123.6 ± 9.8^{a}	39.4 ± 2.9^{a}
ChCl-LA	126.1 ± 6.0^{a}	37.3 ± 3.0^{ab}
Supercritical CO_2	17.5 ± 1.3^{c}	5.2 ± 0.8^{d}

GAE: gallic acid equivalents; QE: quercetin equivalents; ChCl: choline chloride, Et: ethylene glycol; Gly: glycerol; LA: lactic acid. Data are shown as means ± standard deviation of three replicates ($n = 3$). Different superscript letters in the same column indicate a significant difference ($p < 0.05$).

Regarding the total flavonoid content, a behavior similar to the phenolic compounds content was observed, with DES having the highest values. The total flavonoid content in order of extraction was as follows: ChCl-Et, ChCl-Gly, ChCl-LA, methanol, water, and supercritical CO_2, with the choline chloride-ethylene glycol extract having the highest content (39.5 mg QE/g) and supercritical CO_2 the one with the lowest content (5.2 mg QE/g). This behavior can be explained by the high polarity of DES and methanol, which is a

less selective flavonoid extraction; in comparison, the supercritical CO_2 technique is more selective, extracting greater amounts of specific flavonoids that are not easily detected with this colorimetric method [5].

2.2. *Antioxidant Capacity by TEAC, FRAP, DPPH•, and ORAC Assays*

The antioxidant capacity was determined by different methods, and the results are shown in Figure 1. The AC by $ABTS^{\bullet+}$ consists of the coloration intensity reduction of the $ABTS^{\bullet+}$ radical when reacting with an antioxidant compound. It ranged between 311.6 and 1090.9 µmol TE/g sample (Figure 1). The highest values were found in the ChCl-Gly, ChCl-LA, and ChCl-Et extracts (1090.9, 1074.4, and 1028.1 µmol TE/g), respectively, with no significant difference between them. The methanolic extract also showed high antioxidant potential with an antioxidant capacity of 801.6 µmol TE/g, followed by the water extract and supercritical CO_2 (427.9 and 311.6 µmol TE/g), respectively, with significant differences between these extracts. The extract's antioxidant capacity is associated with the composition and content of bioactive compounds in the extracts [14].

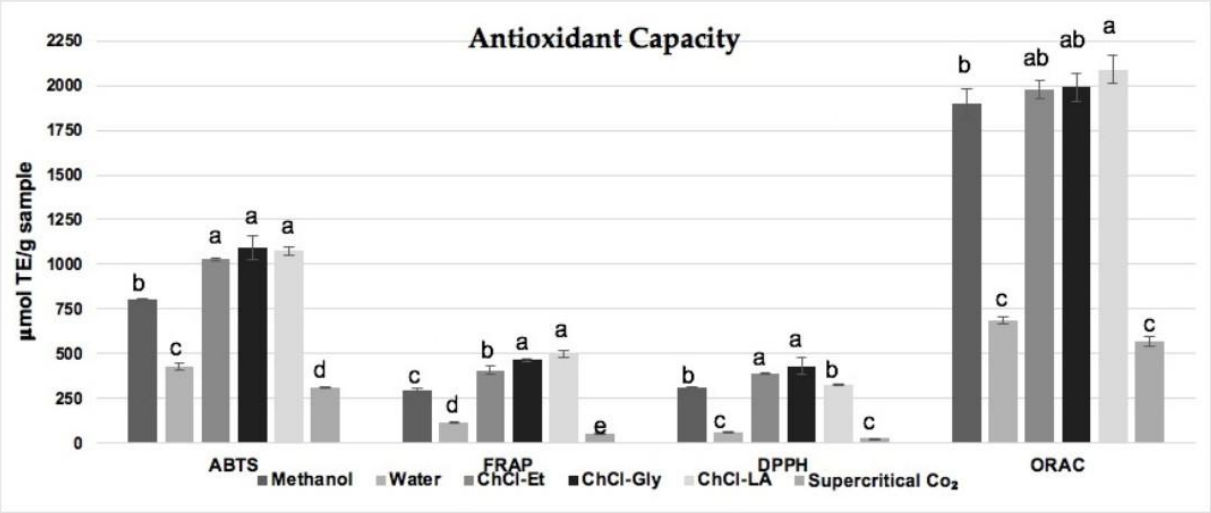

Figure 1. Antioxidant capacity of different oregano extracts (*Lippia graveolens*) by $ABTS^{\bullet+}$, FRAP, $DPPH^{\bullet}$, and ORAC assays. Distinct letters in the same assay bars show the significant difference ($p < 0.05$).

Regarding the results by FRAP, which consists of the reduction of the ferric ion, a similar trend was observed in antioxidant capacity by $ABTS^{\bullet+}$. The ChCl-LA extract (500.2 µmol TE/g) was the one that presented the highest antioxidant capacity, followed by the ChCl-Gly, ChCl-Et, methanol, water, and supercritical CO_2 extracts (464.5, 407.2, 297.8, 118.2, and 56.3 µmol TE/g) respectively. The reducing power is related to the hydroxylation and conjugation degree of bioactive compounds. Therefore, DES's high antioxidant capacity can be explained by their intermolecular interactions, mainly by hydrogen bonds between flavonoids and phenolic acids found in the solvent and the oregano extracts [15,16].

The $DPPH^{\bullet}$ assay was also performed, one of the most widely used assays in plant extracts, where an antioxidant with a weak A–H bond reacts with a free radical $DPPH^{\bullet}$ causing discoloration of the molecule [17]. This study showed the highest values in the ChCl-Gly, ChCl-Et, and ChCl-LA extracts (432.4, 391.3, and 327.4 µmol TE/g), respectively. These DES were reported by Alsaud et al. [18] as the solvents that provide the extract with higher antioxidant capacities. However, the methanolic extract also presented a high antioxidant capacity (311.3 µmol TE/g). On the other hand, the lowest results were observed in water (63.4 µmol TE/g) and supercritical CO_2 (27.5 µmol TE/g) extracts. The

low results in supercritical CO_2 may be due to the dilution factor since the results are expressed in relation to the weight of the plant sample. For this technology, the sample weight is greater than the different methods.

The ORAC assay results, which measures the oxidative degradation of a fluorescent molecule induced by a generator of peroxyl radicals [19], were the following: ChCl-LA presented the highest antioxidant capacity (2090 μmol TE/g) while supercritical CO_2 presented the lowest antioxidant capacity with 571.1 μmol TE/g. However, the methanolic extract showed a high antioxidant capacity (1899.9 μmol TE/g), with no significant difference with the ChCl-LA, ChCl-Gly, and ChCl-Et extracts. These results are very important since this method uses peroxyl radicals, the best model of antioxidant reactions with reactive oxygen species in vivo. This method provides continuous generation of radicals on a realistic time scale [20].

2.3. Identification and Quantification of Oregano (Lippia graveolens) Flavonoid Extracts by UPLC TQS-MSMS

Flavonoids were identified according to the fragments obtained in each sample spectrum with the spectra provided by standards. Sixteen flavonoid-type compounds were searched, of which fourteen were identified. In the methanolic extract, thirteen compounds were identified, eleven in the water extract, and nine in the supercritical CO_2 extract, while in ChCl-Et, ChCl-Gly, and ChCl-LA extracts, eight, twelve, and nine compounds were detected, respectively. Three flavanones were found in our samples, namely naringenin, naringin, and hesperidin; the flavones vitexin, apigenin, luteolin, and luteolin 7-glucoside were also identified. Four flavonoids are flavonols: quercetin, quercitrin, rutin, and kaempferol; two dihydrochalcones identified as phloretin and phloridzin; and one isoflavone identified as genistein (Table 2). The presence of flavonoids in oregano, mostly flavones and flavanones such as apigenin, luteolin-7-glucoside, and naringenin, has been previously reported [21–23]. The quantification of flavonoids was carried out based on commercial standards. The compounds with the highest concentration for all types of extraction/solvent were quercetin, luteolin-7-glucoside, and hesperidin. Standing out with high concentrations were the flavanone, naringenin, and the dihydrochalcone, phloridzin. It is worth mentioning that naringenin has been previously described as predominant in *Lippia graveolens* extracts [5]. However, the flavonoid phloridzin has not been reported as a representative in oregano but in apple tree bark [24,25]. Anti-inflammatory, antioxidant, antidiabetic, and anticancer properties, among others, have been attributed to these compounds [26–28]. It is important to highlight that the methanolic extract was the one that obtained the best flavonoid profile, so it was decided to microencapsulate this extract.

Table 2. Identification and quantification of flavonoids in oregano (*Lippia graveolens*) extracts.

Compounds	Compound Type	Method/Solvent					
		Maceration/ Methanol	Maceration/ Water	Ultrasound-Assisted/ ChCl-Et	Ultrasound-Assisted/ ChCl-Gly	Ultrasound-Assisted/ ChCl-LA	Supercritical/ CO_2-Ethanol
Quercetin	Flavonol	290.04 ± 2.88 ^{b}	8.92 ± 0.91 ^{c}	3.54 ± 0.85 ^{d}	9.93 ± 3.16 ^{c}	2.60 ± 1.61 ^{d}	432.69 ± 0.14 ^{a}
Vitexin	Flavone	ND ^{b}	ND ^{b}	ND ^{b}	1.24 ± 0.63 ^{a}	1.30 ± 0.09 ^{a}	ND
Apigenin	Flavone	15.58 ± 3.05 ^{b}	1.03 ± 0.25 ^{c}	ND ^{c}	ND ^{c}	ND ^{c}	22.22 ± 3.31 ^{a}
Quercitrin	Flavonol	12.57 ± 2.03 ^{ab}	4.59 ± 0.70 ^{c}	13.86 ± 1.45 ^{a}	10.05 ± 1.33 ^{b}	4.66 ± 0.88 ^{c}	ND ^{d}
Luteolin	Flavone	181.28 ± 36.69	26.79 ± 3.73	ND	3.58 ± 0.80	ND	7.63 ± 1.06
L7G	Flavone	1247.84 ± 68.73 ^{b}	489.54 ± 15.09 ^{e}	1334.14 ± 6.78 ^{a}	664.22 ± 21.29 ^{d}	765.73 ± 17.83 ^{c}	1.15 ± 0.26 ^{f}
Naringenin	Flavanone	3505.74 ± 163.60 ^{a}	452.48 ± 22.21 ^{b}	430.31 ± 3.46 ^{b}	286.85 ± 72.07 ^{b}	102.33 ± 21.13 ^{c}	3513.78 ± 113.69 ^{a}
Naringin	Flavanone	1.00 ± 0.10 ^{b}	ND ^{b}	1.50 ± 0.36 ^{b}	1.49 ± 0.24 ^{b}	4.77 ± 1.42 ^{a}	ND ^{b}
Genistein	Isoflavone	15.44 ± 1.91 ^{b}	ND ^{c}	ND ^{c}	ND ^{c}	ND ^{c}	24.03 ± 3.44 ^{a}
Rutin	Flavonol	2.37 ± 0.92 ^{de}	5.25 ± 0.09 ^{d}	36.95 ± 2.61 ^{a}	30.01 ± 1.19 ^{b}	11.24 ± 3.48 ^{c}	ND ^{e}
Hesperidin	Flavanone	25.56 ± 3.58 ^{b}	11.70 ± 2.64 ^{bc}	101.65 ± 2.94 ^{a}	85.81 ± 2.65 ^{a}	81.56 ± 31.56 ^{a}	ND ^{c}
Kaempferol	Flavonol	12.11 ± 2.40 ^{ab}	9.83 ± 0.27 ^{b}	ND ^{d}	2.56 ± 0.04 ^{c}	ND ^{d}	14.22 ± 1.5 ^{a}
Phloretin	Dihydrochalcone	6.26 ± 0.17 ^{b}	7.50 ± 0.23 ^{b}	ND ^{c}	1.61 ± 0.32 ^{c}	ND ^{c}	37.78 ± 2.46 ^{a}
Phloridzin	Dihydrochalcone	4068.91 ± 50.59 ^{a}	1306.56 ± 49.05 ^{d}	3049.26 ± 80.93 ^{b}	1773.93 ± 23 ^{c}	1177.2 ± 100.32 ^{e}	73.61 ± 8.26 ^{f}

ChCl: choline chloride; Et: ethylene glycol; Gly: glycerol; LA: lactic acid; ND: not detected. Results are expressed in micrograms per gram of dry sample (μg/g). Results are shown as means ± standard deviation of three replicates ($n = 3$). Different superscript letters in the same row indicate the significant difference ($p < 0.05$).

2.4. Total Phenolic and Total Flavonoid Content in Oregano Microcapsules

The total phenolic and total flavonoid content in the oregano microcapsules were 17.3 mg GAE/g and 5.0 mg QE/g of sample, respectively, as compared to the non-microencapsulated extract, which showed 83.7 mg GAE/mg and 25.1 mg QE/mg dry extract content, respectively (Table 3). It should be noted that encapsulation efficiencies of 94.5% phenolic compounds and 90.1% total flavonoids were achieved. However, the phenolic content results in the microcapsules were higher than those reported by Bernal-Millan et al. [29]. They obtained 14.05 mg GAE/g of microcapsules, starting from oregano (*Lippia graveolens*) aqueous extract under the same operating conditions. Additionally, other studies, such as the one from Rezende et al. [30], reported the total phenolic compounds and flavonoids (10.16 mg GAE/g and 2.26 mg QE/g, respectively) content of *Malpighia emarginata* DC bioactive compounds microencapsulated by a spray dryer. These results were lower than those reported in our study. The values reported in the oregano microcapsules are lower than those found in the extract without microencapsulation. However, it has been reported that these differences are due to the dispersion of the spatial distribution of the polyphenols when adding an encapsulating polymer; therefore, the quantification per unit of mass decreases [31,32]. In addition, it has been shown that the microencapsulation of bioactive compounds, due to the decrease in water activity, can improve the target compounds with new physical characteristics or simply protect their physicochemical properties, ensure microbiological stability, and avoid degradation processes; it also reduces storage costs and allows a controlled release of the encapsulated active compound [29,33].

Table 3. Total phenolic compounds, flavonoids, and antioxidant capacity $ABTS^{\bullet+}$, FRAP, and ORAC from oregano (*Lippia graveolens*) extract and microcapsules.

Sample	TPC (mg GAE/g Sample)	TFC (mg QE/g Sample)	$ABTS^{\bullet+}$ (μmol TE/g Sample)	FRAP (μmol TE/g Sample)	ORAC (μmol TE/g Sample)
Microcapsules	17.3 ± 2.1	5.0 ± 0.3	241.9 ± 4.7	178.6 ± 2.6	637.0 ± 4.6
Non-microencapsulated extract	83.7 ± 5.9	25.1 ± 2.9	1198.6 ± 55.5	992.6 ± 20.8	4153.8 ± 133.1

TPC: total phenolic content; TFC: total flavonoid content; GAE: gallic acid equivalents; QE: quercetin equivalents; TE: Trolox equivalents. Data shown as means ± standard deviation of three replicates ($n = 3$).

2.5. Antioxidant Capacity by $ABTS^{\bullet+}$, FRAP, and ORAC Assay in Oregano Microcapsules

Table 3 shows the oregano microcapsule's antioxidant capacity values through the $ABTS^{\bullet+}$, FRAP, and ORAC tests. The results were 241.9, 178.6, and 637.0 μmol TE/g sample, respectively. These values were lower than those observed in the extract without microencapsulation for the three types of antioxidant activity assay, which is justified by the dilution of the extract when mixed with the wall material. In addition, the drying process did not affect the estimated antioxidant activity in the microcapsule, observing similar values to those expected.

Regarding the oregano bioactive compounds microencapsulated by spray drying, Bernal-Millan et al. [29] reported 50.83 μmol TE/g and 85.17 μmol TE/g for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals' inhibition. Our high values can be attributed to some modifications in the extraction methods, concentrating the extract, and therefore the microcapsules presented a greater antioxidant capacity. In other studies of the microencapsulation of bioactive compounds, Batista et al. [34] microencapsulated soy molasses isoflavones by spray drying using similar conditions to our research (Maltodextrin 18%, input temperature 140 °C), determining in the microcapsules an antioxidant capacity of 22.74 μmol TE/g through the ORAC assay, this value was much lower than that reported in oregano microcapsules. Additionally, Ydjeed et al. [35] encapsulated phenolic compounds from Carob (*Ceratonia siliqua* L.) pulp extracts, evaluating ORAC, FRAP, and $DPPH^{\bullet}$ capacity, reporting 295.8, 156.1, and 309.7 μmol TE/g, respectively, values below those obtained in the reported oregano microcapsules. These differences between the reported antioxidant capacity values

can be attributed to the bioactive compound type and amount, source type (extract), process type, and encapsulating agent used.

2.6. Identification and Quantification of Flavonoids in Oregano (Lippia graveolens) Microcapsules

The chromatographic profile of the microencapsulated flavonoids and the extract without microencapsulating was determined. The results are shown in Table 4. Ten flavonoids were identified, four from the flavone type, two flavanones, two flavonols, one isoflavone, and one dihydrochalcone. However, there were three major compounds, in order of concentration, naringenin (1181.3 μg/g), phloridzin (677.9 μg/g), and quercetin (329.4 μg/g). These flavonoids have already been reported as the majority compounds except for phloridzin, which its presence has been associated with other matrixes such as apple trees [24]. Bernal-Millan et al. [29] identified 11 flavonoids in oregano (*Lippia graveolens*) microencapsulated phenolic extract and reported among its main compounds naringenin with 204.65 μg/g, reported that the value was five-times lower than that obtained in this study. However, these differences may be influenced by factors such as the sample nature and the fact that they did not use standards for their quantification analysis, reporting all its compounds in quercetin equivalents. Oregano microcapsules have great bioactive potential since they contain flavonoids with antioxidant, anti-inflammatory, diabetic, anticancer, and antihypertensive properties, among others.

Table 4. Identification and quantification of flavonoids in oregano (*Lippia graveolens*) microcapsules.

Compounds	Compound Type	Microcapsules (μg/g)	Non-Microencapsulated Extract (μg/g)
Quercetin	Flavonol	329.43 ± 28.73	1916.0 ± 164.61
Vitexin	Flavone	ND	ND
Apigenin	Flavone	22.40 ± 2.21	53.23 ± 7.34
Quercitrin	Flavonol	ND	ND
Luteolin	Flavone	17.29 ± 0.58	64.24 ± 1.18
Luteolin-7-glucoside	Flavone	67.48 ± 1.74	111.63 ± 8.47
Naringenin	Flavanone	1181.35 ± 104.23	3840.11 ± 199.92
Naringin	Flavanone	ND	ND
Genistein	Isoflavone	15.00 ± 2.70	80.25 ± 0.97
Rutin	Flavonol	3.04 ± 0.15	8.54 ± 0.73
Hesperidin	Flavanone	3.49 ± 0.58	10.09 ± 0.46
Kaempferol	Flavonol	17.33 ± 1.66	82.11 ± 4.54
Phloretin	Dihydrochalcone	ND	ND
Phloridzin	Dihydrochalcone	677.92 ± 6.72	1202.08 ± 35.44

ND: not detected. Results are shown as means ± standard deviation of three replicates (n = 3).

Moreover, the bioactive benefits of polyphenols highly depend on their bioaccessibility and bioavailability, and the main reason for microencapsulation techniques is to increase the stability of polyphenols and enhance their bioaccessibility. Further studies should be focused on the release kinetics of polyphenols from Mexican oregano from these samples and their stability during the gastrointestinal tract.

2.7. Particle Morphology

Size and shape are the main characteristics to consider when producing microcapsules. The micrographs of the oregano microcapsules obtained by scanning electron microscopy showed a particle size between 2 and 10 μm and a spherical shape with flattening (Figure 2). In a study by Bernal-Millán et al. [29], oregano phenolic compounds extracted using water as a solvent were microencapsulated. They found similar features, the particles presented a spherical shape with depressions, and they mentioned that the roughness was attributed to the high drying speed, causing contractions in the microparticles due to the drastic moisture loss. They also obtained particles with sizes between 2 and 12 μm; these dimensions are typical of microcapsules obtained by spray drying. They stated that the

degree of hydrolysis of maltodextrin and the pressure of the atomizing air influence the characteristics of the particles.

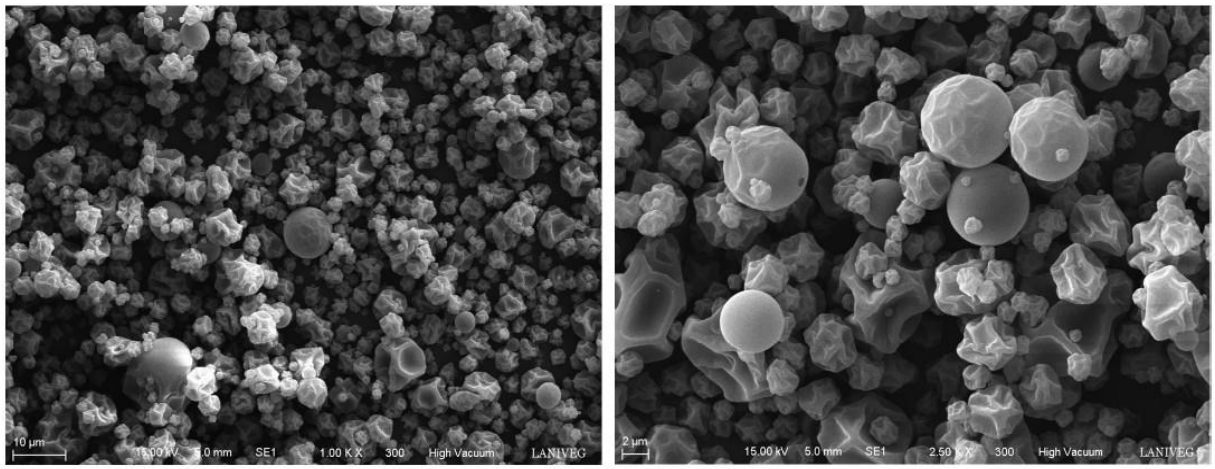

Figure 2. Micrographs of the microparticle structure of oregano (*Lippia graveolens*) flavonoids produced with maltodextrin 10 DE as wall material, using spray drying.

3. Materials and Methods

3.1. Chemicals and Raw Materials

The methanol was obtained from CTR Scientific. The carbon dioxide 99.9% (CO_2) was bought in Lynde, Mexico. The choline chloride, glycerol, ethylene glycol, and lactic acid were obtained from Sigma Aldrich. The analytic standards quercetin (≥95%), vitexin (≥95%), apigenin (≥95%), quercitrin (88.4%), luteolin (≥98%), luteolin-7-glucoside (≥98%), naringenin (≥95%), naringin (≥95%), genistein (≥98%), rutin (94%), hesperidin (≥80%), kaempferol (≥97%), phloretin (≥99%), and phloridzin (≥99%) were from Sigma Aldrich (St. Louis, MO, USA). The mass spectrometry grade acetonitrile was acquired from JT Baker (Phillipsburg, NJ, USA). The oregano was obtained from the indigenous town Temohaya, at Mezquital, Durango, México (N: 23.299722; W: 104.509167). Oregano leaves were dried at 40 °C for 24 h in an Excalibur Food Dehydrator Parallax Hyperware (Sacramento, CA, USA) and minced in an IKA Werke M20 mill (Wilmington, NC, USA), obtaining a fine powder with a sieve #40. The oregano powder was stored at −20 °C until use.

3.2. Extraction of Flavonoids from Oregano (Lippia graveolens)

3.2.1. Extraction of Flavonoids by Maceration

The rich flavonoid extracts were separated following the Chang et al. [36] methodology: 1 g of dried oregano and 10 mL of solvent (methanol and distilled water) were homogenized with an agitator SHAKER DOS-10L (Riga, Latvia) for 2 h in darkness. Subsequently, the extract was centrifuged at 10,000 rpm for 15 min at 4 °C; the supernatant was collected and stored at 4 °C for later use.

3.2.2. Ultrasound-Assisted Extraction of Flavonoids

The extraction solvents were deep eutectic solvents (DES), synthesized according to Barbieri et al. [12], with some modifications. Choline chloride (ChCl) was used as hydrogen bond acceptor, and ethylene glycol (Et), glycerol (Gly), and lactic acid (LA) as hydrogen bond donors; the reagents were mixed and heated to 50 °C with 30% water. The synthesized DES were ChCl-Et, ChCl-Gly, and ChCl-LA at a 1:4 molar ratio. For the flavonoid extraction, 1 g of dried oregano and 10 mL of solvent (ChCl-Et, ChCl-Gly, ChCl-LA) were homogenized in an agitator. Subsequently, they were sonicated in a Branson ultrasonic CPX8800H-E (Brookfield, CT, USA) for 45 min at 45 °C and 40 kHz. Finally, the obtained extract was

centrifuged at 10,000 rpm for 15 min at 4 °C; the supernatant was collected and stored at 4 °C for later use.

3.2.3. Extraction of Flavonoids by Supercritical Fluid

The flavonoids were extracted following the protocol of Picos-Salas et al. [5]. An MV-10 ASFE (Waters Corp., Milford, MA, USA) extractor was used with CO_2 as solvent and ethanol (99.9%) as a cosolvent in a 95:5 ratio. First, 2.5 g of sample were placed in a 10 mL beaker, and the following conditions were used: CO_2 flow (4.75 mL/min), ethanol flow (0.25 mL/min), pressure at 245 bar, temperature at 36 °C, and static and dynamic extraction time (30 min, each). After the extraction, the extract was dried in a vacuum concentrator. Then, the yield was calculated, and the sample was resuspended in ethanol (99.9%) and stored at −20 °C for further analysis.

3.3. Antioxidant Analysis

3.3.1. Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay determined the antioxidant capacity is based on absorbance inhibition of the $ABTS^{\bullet+}$ radical caused by the reaction with antioxidants, following the Thaipong et al. [37] methodology. The reaction solution was prepared by homogenizing 1 mL of 2.6 mM potassium persulfate and 1 mL of 7.4 mM $ABTS^{\bullet+}$. The mixture was left at room temperature for 16 h. For the assay, 10 µL of the extract was added to 190 µL of the reaction solution ($ABTS^{\bullet+}$). Subsequently, it was incubated in darkness for 2 h. Finally, the plate was read at 734 nm in a Synergy HT Microplate reader (BioTek, Inc., Winooski, VT, USA). Results were expressed in equivalent micromoles of Trolox per gram dry sample (µmol TE/g).

3.3.2. Oxygen Radical Absorbance Capacity (ORAC)

For this test, the methodology of Huang et al. [38] was followed. Fluorescein was used as a fluorescent probe, Trolox as standard, and AAPH (2,2′-azobis dihydrochloride (2-amidino-propane)) as a peroxyl radical generator. The reaction mixture included 25 µL of extract, 75 µL of AAPH 95.8 µM, and 200 µL of 0.96 µM fluorescein in a 96-well microplate with a clear bottom and black walls; 75 mM phosphate buffer was used as blank. The fluorescein, samples, and phosphate buffer were preincubated at 37 °C for 15 min. Fluorescence loss was measured every 70 s for 70 min at a 485 nm wavelength for excitation and 580 nm for emission using a Synergy HT spectrophotometer. Values were calculated using a regression equation that describes the relationship between Trolox concentration and the net area under the fluorescein decomposition curve. To calculate the results, a Trolox curve from 6.25 to 125 (µmol TE/g) was used, and the results are expressed in equivalent µmol of Trolox per gram dry sample (µmol TE/g).

3.3.3. Antioxidant Capacity by $DPPH^{\bullet}$ Assay

This method uses the radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and reduces its purple chromogen by the action of an antioxidant compound to hydrazine, which colors a pale-yellow tone. The test was executed according to what was described by Karadag et al. [39]. First, in a 96-well plate, 10 µL of extract, 10 µL of Trolox standard curve, and 10 µL of blank (solvent) were added; then 190 µL of $DPPH^{\bullet}$ 200 µM reagent was added. It was incubated in darkness for 30 min. Finally, samples in the plate were read at 540 nm absorbance in a Synergy HT Microplate Reader (BioTek, Inc., Winooski, VT, USA). Results are expressed in equivalent µmol of Trolox per gram dry sample (µmol TE/g).

3.3.4. Ferric Reducing Antioxidant Power Assay (FRAP)

This analysis is based on the power of a compound or an extract to reduce the ligand complex of ferric ions (Fe^{3+}) to the deep blue-colored ferrous complex (Fe^{2+}) in an acid medium. The FRAP analysis was performed as detailed by Ghasemzadeh et al. [40]. First, the FRAP reagent was prepared by mixing 1 mL of TPTZ 30 mM, 1 mL of $FeCl_3 \bullet 6H_2O$

60 Mm, and 10 mL of acetate buffer. Then, in a 96-well plate, 30 µL of extract, 30 µL of Trolox standard curve, and 30 µL of blank (solvent) were added. 120 µL of FRAP reagent was added. It was incubated in darkness for 4 min. Finally, samples in the plate were read at 590 nm absorbance in a Synergy HT Microplate Reader (BioTek, Inc., Winooski, VT, USA). Results are expressed in equivalent µmol of Trolox per gram dry sample (µmol TE/g).

3.4. Total Phenolic and Flavonoid Content

3.4.1. Total Phenolic Content

The total phenolic content, also known as total reducing capacity, was done to measure the total phenol content determined by the Folin–Ciocalteu reagent, following the methodology reported by Swain and Hillis et al. [41], with some modifications. First, the reaction mixture was prepared by mixing 10 µL of the sample, 230 µL of distilled water, 10 µL of Folin-Ciocalteu reagent, and 25 µL (2 M) of sodium carbonate solution. The reaction mixture was incubated for 2 h before absorbance was read at 725 nm using a Synergy HT 96-well microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The results were expressed in equivalent milligrams of gallic acid per gram dry sample (mg GAE/g).

3.4.2. Total Flavonoid Content

As reported by Chang et al. [36], the aluminum chloride colorimetric method determined total flavonoid content with slight modifications. First, 10 µL of the prepared extract, 10 µL of a blank (extraction solvent), and 10 µL of a quercetin standard curve were added to a 96-well plate. Subsequently, 250 µL of distilled water, 10 µL of 10% aluminum chloride, and 10 µL of 1 M potassium acetate were added. The sample was incubated in darkness for 30 min before reading the plate in a Synergy HT Microplate reader (BioTek, Inc., Winooski, VT, USA) at an absorbance of 415 nm. The results were reported in equivalent milligrams of quercetin per gram dry sample (mg QE/g).

3.5. Identification and Quantification of Flavonoids by UPLC-TQS-MS/MS

The identification and quantification of oregano flavonoids were carried out following the report by Bernal-Millan et al. [29] with some modifications, using an UPLC class H equipment (Waters Corp., Milford, MA, USA) coupled to a XEVO-TQS mass analyzer (Triple Quadrupole) using a BeH Phenyl 1.7 mm × 2.1 × 100 column at 40 °C. The compounds were separated with an elution gradient, solution A (water-ammonium formate 5 mM pH 3) and solution B (acetonitrile-0.5% formic acid) at a 0.3 mL/min flow rate. The procedure for gradient elution was as follows: 0 min, 90% (A); 5 min, 10% (A); 5.10 min, 90% (A); and 8 min, 90% (A). Electrospray ionization (ESI) was performed to analyze compounds; the parameters used were a 1.5 kV capillary voltage, a 30 V sampling cone, temperature at 500 °C, and desolvation gas 800 (L/h). The identification and quantification of flavonoids by UPLC were performed based on the retention time and wavelength peak area of maximum absorption. Multilevel calibration curves were performed using the standards apigenin, vitexin, quercetin, quercitrin, luteolin, luteolin-7-glucoside, naringin, phloretin, phloridzin, kaempferol, hesperidin, rutin, naringenin, and genistein (Figure S1). The flavonoid content was expressed in micrograms per gram dry sample.

3.6. Preparation of Oregano (Lippia graveolens) Microcapsules

Firstly, as previously reported, the extraction of oregano flavonoids was carried out by maceration using methanol as a solvent. Then, the extract was evaporated in a rotary evaporator; the dry extract was resuspended in 100 mL of distilled water. Subsequently, the feed solution was prepared. The 100 mL of extract (3.5 g of dry extract) were mixed with 16% maltodextrin 10 DE on a stirring plate (Thermo Scientific Cimarec, Waltham, MA, USA) until completely dissolved. The mixture was fed to a Yamato AD311S Spray Dryer with the following conditions: inlet temperature at 145 °C, feed flow of 5 mL/min, airflow at 0.32 m^3/min, and an atomization pressure of 0.1 MPa. The microcapsules were recovered and stored for later analysis.

3.7. Flavonoid Encapsulation Efficiency and Particle Morphology

The encapsulation efficiency was calculated using the ratio between the theoretical flavonoid and the flavonoid content obtained in the microcapsules and reported as a percentage. Microparticle morphology was analyzed by an environmental scanning electron microscope model EVO-50 (Carl Zeiss, Oberkochen, Germany). The sample was placed using double-sided adhesive carbon tape on a sample holder without previous treatment. Observation was made with a secondary electron detector (SE1) and a 10–15 kV acceleration voltage ($\times$2000 and $\times$4000 magnification) under high vacuum conditions.

3.8. Statistical Analysis

All experiments had three replicates. Data were expressed as the mean $\pm$ standard deviation (SD). Significant differences in evaluated parameters among different samples were analyzed by Minitab software (version 21.0, Minitab LLC, State College, PA, USA). The probability value of $p < 0.05$ was considered significant.

4. Conclusions

It was found that both conventional methods and emerging extraction technologies of bioactive compounds such as flavonoids present high extraction yields; however, the methanol-maceration method and the ultrasound-assisted extraction-DES showed the best antioxidant results. Microencapsulation of oregano flavonoids was also carried out, achieving a 90.1% encapsulation; various tests determined that the microcapsules presented antioxidant potential. Additionally, 14 flavonoids were identified and quantified, highlighting naringenin and phloridzin, reported with high bioactive potential. For all the above, the different oregano (*Lippia graveolens*) extracts, which are rich in flavonoids, and the microcapsules present promising results to investigate their potential against various diseases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12081692/s1, Figure S1: UPLC-MS chromatograms for flavonoid standards.

Author Contributions: Conceptualization, M.d.J.B.-M. and J.B.H.; methodology, M.d.J.B.-M. and P.d.J.B.-B.; validation, E.P.G.-G. and J.B.H.; formal analysis, M.d.J.B.-M. and P.d.J.B.-B.; investigation, M.d.J.B.-M., M.d.C.C.-P. and M.Á.A.-E.; resources, J.B.H.; writing—original draft preparation, M.d.J.B.-M.; writing—review and editing, E.P.G.-G., J.L.-F. and J.B.H.; visualization, M.d.C.C.-P., J.L.-F. and M.Á.A.-E.; supervision, J.B.H.; project administration, J.B.H.; funding acquisition, J.B.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data are included in the main text.

Acknowledgments: Thanks to the Consejo Nacional de Ciencia y Tecnología (CONACYT) México for providing a scholarship to Bernal-Millán (#633460). We appreciate the technical support of Laura Contreras-Angulo.

Conflicts of Interest: The authors state that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3. ANTI-INFLAMMATORY EFFECT OF FREE AND MICROENCAPSULATED EXTRACT OF *Lippia graveolens* IN A CARRAGEENAN–INDUCED PAW EDEMA MODEL AND AN OVALBUMIN INDUCED ASTHMA MODEL IN MICE (ENVIADO)

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Revista:

Journal of Ethnopharmacology

Fecha de envío:

18 de diciembre del 2024

Numero de manuscrito:

JETHNO-D-24-12192

Journal of Ethnopharmacology

Anti-Inflammatory Effect of Free and Microencapsulated Extract of Lippia graveolens in a Carrageenan-Induced Paw Edema Model and an Ovalbumin-Induced Asthma Model in Mice

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Paper
Keywords:	Inflammation; Lippia graveolens; paw edema; allergic asthma; airway inflammation; polyphenols
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Abstract:	Ethnopharmacological relevance Lippia graveolens has been widely used in traditional Mexican medicine to relieve stomach pain, menstrual pain, and respiratory illnesses (flu and cough). However, its anti-inflammatory effect has not been studied in acute and chronic inflammation models. Aim of the study to evaluate the effect of L. graveolens extract and microcapsules against paw and airway inflammation. Materials and methods Paw edema in mice was induced with carrageenan, while allergic asthma was sensitized and challenged with ovalbumin. L. graveolens samples were orally administered to mice in 30, 100, 300, 560, and 717 mg/kg doses to evaluate edema size, while for the following tests, the 560 mg/kg dose was administered. Inflammatory cell count, serum IgE level, and detection of proinflammatory cytokine levels in serum, plantar tissue, and lung tissue were analyzed. Results Edema thickness measurement revealed that L. graveolens reduces paw inflammation and could potentially be anti-inflammatory in acute and chronic inflammation. Treatments with L. graveolens significantly decreased the number of inflammatory cells in the blood, the production of IgE in serum, and the cytokines IL-1β, IL-6, and TNF-α levels in serum, paw, and lungs. Conclusions

	These findings suggest that L. graveolens has an anti-inflammatory effect on carrageenan-induced paw edema and ovalbumin-induced allergic asthma in mice and might be a promising protective agent for inflammatory diseases.
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A.M. Viljoen, PhD
Editor-in-Chief
Journal of Ethnopharmacology

Dear Editor,

We are pleased to submit an original research article titled "Anti-inflammatory effect of free and microencapsulated extract of *Lippia graveolens* in a carrageenan-induced paw edema model and an ovalbumin-induced asthma model in mice" to be considered for publication in the Journal of Ethnopharmacology.

The ethnopharmacological relevance of this manuscript is mainly related to the broad use of *L. graveolens* in traditional Mexican medicine to relieve stomach pain, menstrual pain, and respiratory illnesses (flu and cough). However, its anti-inflammatory effect has not been studied in acute and chronic inflammation models. Therefore, in this study we aim to evaluate the effect of *L. graveolens* extract and microcapsules against paw and airway inflammation.

Briefly, paw edema in mice was induced with carrageenan, while allergic asthma was sensitized and challenged with ovalbumin. *L. graveolens* samples were orally administered to mice in 30, 100, 300, 560, and 717 mg/kg doses to evaluate edema size, while for the following tests, the 560 mg/kg dose was administered. Inflammatory cell count, serum IgE level, and detection of proinflammatory cytokine levels in serum, plantar tissue, and lung tissue were analyzed. The edema thickness revealed that *L. graveolens* reduces paw inflammation and could potentially be anti-inflammatory in acute and chronic inflammation. Treatments with L. graveolens significantly decreased the number of inflammatory cells in the blood, the production of IgE in serum, and the cytokines IL-1β, IL-6, and TNF-α levels in serum, paw, and lungs.

These findings suggest that *L. graveolens* has an anti-inflammatory effect on carrageenan-induced paw edema and ovalbumin-induced allergic asthma in mice and might be a promising protective agent for inflammatory diseases. We consider our work to be of common interest to the pharmaceutical industry and pharma scientists. We would also like to state that this manuscript has not been published and is not being considered for publication elsewhere. We have no conflicts of interest to disclose. Furthermore, all authors approved this submission.

Thank you for your consideration,

Sincerely,

J. Basilio Heredia, Ph.D.
Miriam C. Carrasco-Portugal, Ph.D.
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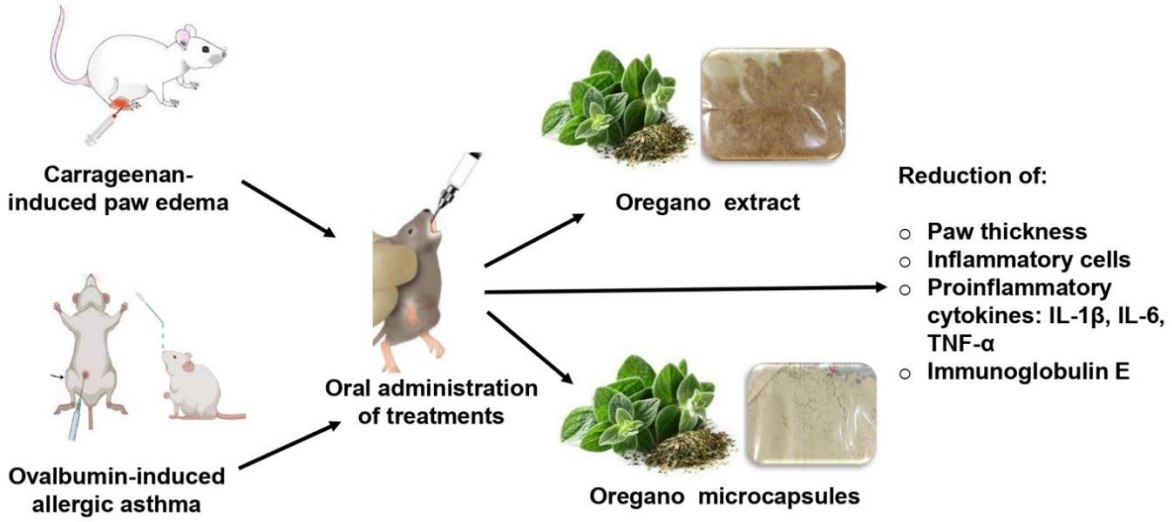
Culiacán, MX, December 18, 2024.

Graphical Abstract



Anti-Inflammatory Effect of Free and Microencapsulated Extract of *Lippia graveolens* in a Carrageenan-Induced Paw Edema Model and an Ovalbumin-Induced Asthma Model in Mice

Manuel J. Bernal-Millán[a], José E. Roa-Coria[b], Francisco J. Flores-Murrieta[b,c], Juan C. Huerta-Cruz[b], Erick P. Gutiérrez-Grijalva[d], Josefina León-Félix[a], Miguel A. Angulo-Escalante[a], J. Basilio Heredia[a*], Miriam C. Carrasco-Portugal[b*]

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Abstract

Ethnopharmacological relevance: *Lippia graveolens* has been widely used in traditional Mexican medicine to relieve stomach pain, menstrual pain, and respiratory illnesses (flu and cough). However, its anti-inflammatory effect has not been studied in acute and chronic inflammation models.

Aim of the study: to evaluate the effect of *L. graveolens* extract and microcapsules against paw and airway inflammation.

Materials and methods: Paw edema in mice was induced with carrageenan, while allergic asthma was sensitized and challenged with ovalbumin. *L. graveolens* samples were orally administered to mice in 30, 100, 300, 560, and 717 mg/kg doses to evaluate edema size, while for the following tests, the 560 mg/kg dose was administered. Inflammatory cell count, serum IgE level, and detection of proinflammatory cytokine levels in serum, plantar tissue, and lung tissue were analyzed.

Results: Edema thickness measurement revealed that *L. graveolens* reduces paw inflammation and could potentially be anti-inflammatory in acute and chronic inflammation. Treatments with *L. graveolens* significantly decreased the number of inflammatory cells in the blood, the production of IgE in serum, and the cytokines IL-1β, IL-6, and TNF-α levels in serum, paw, and lungs.

Conclusions: These findings suggest that *L. graveolens* has an anti-inflammatory effect on carrageenan-induced paw edema and ovalbumin-induced allergic asthma in mice and might be a promising protective agent for inflammatory diseases.

Keywords: Inflammation, *Lippia graveolens*, paw edema, allergic asthma, airway inflammation.

1. Introduction

Inflammation is an immune system protective response against various pathogens, signs of cell damage, and tissue injury (Ramhwati et al., 2022). Inflammation can be acute or chronic, depending on the type of stimulus and the effectiveness of eliminating said stimuli (Ambriz-Pérez et al., 2016). Acute inflammation usually involves pain and swelling at the site of injury due to the release of many proinflammatory mediators and fluid leakage from vascular tissues due to increased permeability of vessel walls, migration of inflammatory cells, tissue damage, and scarring (Gupta et al., 2015). When the immune system fails to eliminate these harmful agents, it can cause a chronic phase. For any phase of inflammation, a pharmacological intervention is required; non-steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, indomethacin, naproxen, and ibuprofen are generally used for acute inflammation (Ou et al., 2019). These inhibit the activities of the enzymes cyclooxygenase-1 and cyclooxygenase-2, preventing the synthesis of thromboxanes and prostaglandins and, therefore, exhibit antipyretic, analgesic, and anti-inflammatory effects. While for chronic inflammation, steroidal anti-inflammatory drugs (SAIDs) are used, including prednisone acetate and dexamethasone acetate; they inhibit inflammatory genes by binding to glucocorticoid receptors and activating anti-inflammatory genes (Semis et al., 2021). However, both NSAIDs and SAIDs have side effects that cause serious health problems. For this reason, alternatives are being sought that may be more effective and safer against inflammation.

In recent years, natural compound sources like oregano have been studied as an alternative due to its phenolic compounds, mainly flavonoids, to which therapeutic properties such as

anti-aging, antihypertensive, antiviral, anticancer, and related to all these, an anti-inflammatory property is attributed (Gutiérrez-Grijalva et al., 2018). The main flavonoids in oregano are naringenin, phloridzin, apigenin, and quercetin, all related to anti-inflammatory properties (Bernal-Millán et al., 2023). The anti-inflammatory effect of phloridzin and naringenin isolated from oregano (*Lippia graveolens*) was studied in mice with ear edema induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), managing to reduce the edema by 42.63% and 41.96%, respectively (Amador et al., 2020). However, one of the most reliable methods to evaluate the anti-inflammatory properties of drugs and natural compounds in acute inflammation is paw edema caused by intraplantar carrageenan injection (Alblihed et al., 2020). In contrast, the ovalbumin-induced asthma mice model is used to evaluate the anti-inflammatory potential in chronic inflammation (Kim et al., 2020). In the present study, the anti-inflammatory properties of oregano-free extracts and microencapsulated extract were investigated against carrageenan-induced paw edema and airway inflammation in an ovalbumin-induced asthma model in mice.

2. Materials and Methods

2.1. Reagents and Chemicals

Diclofenac used in the study was obtained from Colmed International. λ-Carrageenan was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Ovalbumin (OVA) was purchased from Sigma-Aldrich (St. Louis, MO, USA), $Al(OH)_3$ from Tecsiquim (Iztacalco, CDMX, Mexico), dexamethasone from Supelco (Merck KGaA, Darmstadt, Germany). Flavonoid standards were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Oregano extract and microencapsulated extract preparation

The aqueous extract of oregano (*L. graveolens*) was obtained by macerating dried leaves, according to Bernal-Millán et al. (2023). This was lyophilized until a dry extract was obtained. The oregano microcapsules were obtained by microencapsulating the lyophilized dry extract with maltodextrin by spray drying following Bernal-Millán et al. (2022) methodology.

2.3. Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

The identification and quantification of oregano flavonoids were carried out following the report by Bernal-Millán et al. (2023), with some modifications, using a UPLC class H equipment (Waters Corp., Milford, MA, USA) coupled to a XEVO-TQS mass analyzer (Triple Quadrupole) using a BeH Phenyl 1.7 mm × 2.1 × 100 column at 40°C. The compounds were separated with an elution gradient, solution A (water-ammonium formate 5 mM pH 3) and solution B (acetonitrile-0.5% formic acid) at a 0.3 mL/min flow rate. The procedure for gradient elution was as follows: 0 min, 90% (A); 5 min, 10% (A); 5.10 min, 90% (A); and 8 min, 90% (A). Electrospray ionization (ESI) was performed to analyze compounds; the parameters used were a 1.5 kV capillary voltage, a 30 V sampling cone, temperature at 500°C, and desolvation gas 800 (L/h). Identification and quantification of flavonoids by UPLC were performed using multilevel calibration curves using the standards apigenin, vitexin, quercetin, quercitrin, luteolin, luteolin-7-glucoside, naringin, phloretin, phloridzin, kaempferol, hesperidin, rutin, naringenin, and genistein. The flavonoid content was expressed in micrograms per gram of dry extract.

2.4. Animals

Ethics committee approval for the study was obtained from the Local Ethics Committee for Experimental Animals of the Ismael Cosío Villegas National Institute of Respiratory Diseases (INER) (Approval No: B18-23). The ICR mice were obtained from the INER's Experimental Vivarium, weighed between 20 and 35 g, and cared for at this institute. The environmental conditions where they took refuge were controlled room temperature under a 12-h dark/12-h light cycle. Regarding their diet, they had free access to food and water. Before starting the experiment, the animals adapted to the medium for 7 d.

2.5. Carrageenan-Induced Acute Paw Edema Mouse Model

According to Rahmawati et al. (2022), edema induction was developed with some modifications. Fourty-five mice were randomly divided into five groups: group 1 (Naive), 2 (carrageenan), 3 (diclofenac), 4 (oregano extract), and 5 (oregano microcapsules), with each group including nine mice. To establish the paw edema model, mice were administered an intraplantar injection of 100 µL of 1% carrageenan 30 min after the final administration of the treatments in groups 2, 3, 4, and 5. In contrast, mice from group 1 received an equal volume of phosphate-buffered saline (PBS) without carrageenan.

For oral treatment, the doses were administered as follows: 1, 3, 10, 56, and 100 mg/kg diclofenac (group 3); 30, 100, 300, 560, and 717 mg/kg oregano extract (group 4); and, 30, 100, 300, 560 and 717 mg/kg oregano microcapsules (group 5). Mice in groups 1 and 2 were treated orally with PBS. After evaluating the thickness of the right paw for 4 h, the best dose of each treatment was selected.

2.6. Ovalbumin-Induced Asthma Mouse Model

Sensitization was developed according to the methodology of Wei et al., 2016, with some modifications. Thirty mice were divided into five groups: group 1 (Naive), 2 (OVA), 3 (dexamethasone), 4 (oregano extract), and 5 (oregano microcapsules), with each group including six mice. Mice in groups 2, 3, 4, and 5 were sensitized with 20 µg of OVA emulsified in 2 mg aluminum hydroxide in a total volume of 200 µL of PBS by an intraperitoneal (i.p.) injection at 0, 7, and 14 d.

As a challenge test, mice were sensitized with 100 µg of OVA in 50 µL of PBS by intranasal administration at 25, 26, and 27 d. Also, from day 25-27, 2 mg/kg dexamethasone (group 3), 560 mg/kg oregano extract (group 4), and 560 mg/kg oregano microcapsules (group 5), were orally administered. Mice in group 1 and group 2 were treated orally with PBS. In both sensitization methodologies, mice in group 1 received PBS without OVA at 0, 7, 14, 25, 26, and 27 d.

2.7. Measurement of paw thickness

Paw edema thickness was determined by Stainless Steel-Large LCD vernier digital caliper (Jiavarry, China) just before carrageenan administration and 1, 2, 3, and 4 h afterward. Measurement results are given in micrometers (Laavola et al., 2017).

2.8. Samples collection

After 2 h, blood samples were collected by cardiac puncture under mild anesthesia with isoflurane inhalation. To evaluate serum cytokines, blood samples were collected in a BD SST Microtainer tube without anticoagulant to obtain serum. For this, the blood was centrifuged at 1500 rpm for 10 min. Whole blood was stored in a BD Microtainer® EDTA

K2 tube for the hematology test. The serums were stored at −80°C until biochemical and molecular analysis. Lung tissue (ovalbumin model) and right lower limb tissue (carrageenan model) were also extracted, and these were homogenized in PBS with protease inhibitors (Makni et al., 2019).

2.9. Hematological analysis

Hematological analyses (WBC: white blood cells: monocytes, eosinophils, lymphocytes, basophils, and neutrophils) of blood taken into anticoagulant tubes were performed with an automatic cell counter XN-1000 (Makni et al., 2019; Semis et al., 2021).

2.10. Measurement of cytokine levels in mice serum, plantar area and lungs

Interleukin IL-1β, IL-6, and tumor necrosis factor (TNF)-α were evaluated in serum, plantar area, and lungs by Bio-plex multiplex immunoassay system using a Bio-plex Mouse cytokine kit and quantified on a Bio-Plex 200 flow cytometer according to the manufacturer's protocol. The levels of these cytokines were expressed in picograms per milliliter of sample (Gupta et al., 2015; Ou et al., 2019).

2.11. Total serum IgE measurement

IgE levels were determined with the BIOMED IgE reagent by in vitro quantitative immunological turbidimetric assay of immunoglobulin E in serum, using an AU5800 clinical chemistry analyzer (Beckman Coulter Ireland Inc. Lismeehan, O'Callaghan's Mills Co. Clare, Ireland) (Wijerathne et al., 2017).

2.12 Results analysis and statistics

The time course and dose-effect curve were plotted to evaluate the anti-inflammatory response of the different drugs at the various doses used to determine the effective dose 50 and, at the same time, to determine the most effective dose, both for the oregano extract and the microcapsules. In addition, a one-way ANOVA test was used to evaluate the results of white blood cells and total IgE, followed by a Tukey post-hoc test to determine the statistical significance between more than two groups. The data are expressed as mean ± standard error, and p-values less than 0.05 are considered statistically significant differences.

In the case of cytokine quantification, the Student t-test was used to analyze if there was a significant difference between the two groups (Naive vs OVA or carrageenan). After the normality analysis, the graph shows as: ★ with a $p < 0.05$, ★★ with a $p < 0.01$ and ★★★ with a $p < 0.001$. A one-way ANOVA test was used to analyze if there is a significant difference between more than two groups (OVA or carrageenan vs treatments), followed by a Tukey post hoc test. After the normality and homoscedasticity analysis, the graphs are shown as: + with a $p < 0.05$, ++ with a $p < 0.01$, and +++ with a $p < 0.001$.

3. Results

3.1 Identification and quantification of flavonoids in oregano (L. graveolens) extract and microcapsules

The bioactive compounds were quantified using multilevel calibration standard curves. Eight flavonoids were identified in the oregano extract (lyophilized) and the microencapsulated dry extract (Table 1). Compounds from the flavonol group, such as quercetin, rutin, and kaempferol, were found; flavones such as luteolin and luteolin-7-glucoside; flavanones such as naringenin and hesperidin, as well as a dihydrochalcone known as phloridzin. The

flavonoids identified as the majority in order of concentration were phloridzin, luteolin-7-glucoside, hesperidin, and naringenin, respectively.

Other researchers have previously reported these flavonoids in oregano. However, the high concentration of phloridzin (+15 mg/g of extract) stands out. This is attributed to the extraction and stabilization method, since water was used as a solvent and lyophilization as a drying technique to obtain a stable extract in powder form. It is important to mention that these major compounds have been attributed to anti-inflammatory potential in cell lines and some *in vivo* models (Amador et al., 2020; Chuang et al., 2018; Leyva-López et al., 2022; Vujicic et al., 2015).

Table 1. Flavonoids identification and quantification in oregano extract and microcapsules

Compounds	Oregano extract (µg/g)	Oregano Microcapsules (µg/g)
Quercetin	27.58 ± 2.85	34.07 ± 1.08
Luteolin	47.90 ± 1.98	46.11 ± 0.17
Luteolin-7-glucoside	923.02 ± 14.77	1018 ± 17.13
Naringenin	193.99 ± 14.88	487.55 ± 16.28
Hesperidin	628.68 ± 17.14	817.09 ± 23.86
Rutin	26.47 ± 1.94	31.36 ± 1.06
Kaempferol	25.37 ± 0.81	22.15 ± 0.69
Phloridzin	15,306.33 ± 235.72	15,048.01 ± 377.38

Results are shown as means ± standard deviation of three replicates (n = 3).

3.2 Effects of oregano extract and oregano microcapsules on thickness in carrageenan-induced paw edema in mice

As a result of the acute model, the temporal evolution was evaluated for five different doses. Figure 1 shows the temporal evolution of inflammation at doses of 717 mg/kg of extract and microcapsules. The X-axis shows the time at which the thickness measurements were made, while the Y-axis shows the thickness that the paw gained after administering carrageenan and the treatments. It can be observed that in the 1% carrageenan group, inflammation was already high at 1 h. As time passed, it gradually increased until reaching 2.5 mm of thickness at 4 h because the mice did not receive pharmacological treatment. The naive group (control) was administered only PBS on the paw to identify if it caused inflammation.

Figure 1. Time course of inflammation in a carrageenan-induced paw edema model.

Also, it was observed that inflammation was 0.10 mm at 4 h. The control drug, diclofenac (100 mg/kg), significantly reduced edema after 1 h. A similar behavior was observed with the oregano extract (717 mg/kg), showing a slightly lower but non-significant effect. Likewise, the microcapsules (717 mg/kg) presented the same effect as the control drug.

The diclofenac drug at doses of 1, 3, 10, 56, and 100 mg/kg had a dose-dependent anti-inflammatory effect (Figure 2); the effect obtained by the extract at doses of 30, 100, 300, 560, and 717 mg/kg was also dose-dependent, with the 560 mg/kg dose having the greatest effect, showing non-significant difference with the 717 mg/kg dose.

Figure 2. Anti-inflammatory effect of different doses of diclofenac, oregano extract, and oregano microcapsules on mice with carrageenan-induced paw edema. Significant difference between groups with different letters on the bars.

Regarding the microcapsules (Figure 2), the evaluated doses were 30, 100, 300, 560, and 717 mg/kg, showing a dose-dependent anti-inflammatory effect, with 717 mg/kg being the most effective dose, showing a non-significant difference with the 560 mg/kg dose.

Comparative dose-response curves were performed for the oregano samples and diclofenac as a reference drug (Figure 3). It can be observed that both the oregano extract and the oregano microcapsules have similar behavior; at the same doses, they show similar anti-inflammatory effects. Opposite to this, we observed that diclofenac at lower doses presents the same effect as the oregano extracts.

With these curves, the effective doses 50 (ED50) of the samples were determined and expressed as $ED_{50} \pm$e.e. Diclofenac results showed 51.25$\pm$25.83 mg/kg, while oregano extract 297.36$\pm$51.38* mg/kg, and oregano microcapsules 219.48$\pm$33.04* mg/kg (*$p<0.05$ significant difference concerning the reference drug, by a Student's t-test).

Figure 3. Dose-response curves of diclofenac, oregano extract, and microcapsules on their anti-inflammatory effect in a carrageenan-induced paw edema model.

3.3 Hematology: Treatment effect on leukocytes, lymphocytes, and neutrophils in a carrageenan-induced paw edema model

During the inflammatory process, there is usually an increase in inflammatory cells that migrate to the site of injury, accumulate, and form edema. Table 2 shows the hematological results obtained from the experimental groups. In the carrageenan group, leukocytes

increased about 5 times more than in the Naive group (control: without intervention), which guarantees an efficient edema induction.

Table 2. Leukocytes, lymphocytes, and neutrophils in the blood of mice with carrageenan-induced inflammation.

Group	Leukocytes	Lymphocytes	Neutrophils
Naive	1.62 ± 0.50	1.31 ± 0.35	0.05 ± 0.02
Carrageenan	$7.62 \pm 0.29^{\star}$	$6.49 \pm 0.12^{\star}$	0.36 ± 0.01
Diclofenac (100 mg/kg)	$2.71 \pm 0.89^{+}$	$2.08 \pm 0.62^{+}$	0.21 ± 0.09
Extract (560 mg/kg)	$2.28 \pm 0.33^{+}$	$1.53 \pm 0.25^{+}$	0.18 ± 0.07
Microcapsules (560 mg/kg)	$4.72 \pm 0.46^{+}$	$3.46 \pm 0.47^{+}$	0.44 ± 0.28

Values are expressed in cells ($10^3/mm^3$). * Significant difference between two groups (Naive vs Carrageenan; $p<0.5$). $^{+}$ Significant difference regarding the Carrageenan group ($p<0.05$).

A reduction in leukocyte levels is also observed compared to the carrageenan group, with the administration of diclofenac (81.8%), oregano extract (89%), and oregano microcapsules (48.5%), with no significant difference between the group administered with extract and the Naive group, this indicates a high anti-inflammatory efficacy of the oregano extracts.

In addition, in the differential count, lymphocytes were reduced by 95.8 and 58.5% (significant difference compared to the carrageenan group), with the administration of extract and microcapsules, respectively. While the lyophilized extract reduced the neutrophil content by 58%, there were no significant differences compared to the inflammation group.

In addition, in the differential count, lymphocytes were reduced by 95.8 and 58.5% (showing significant difference compared to the carrageenan group) with the administration of extract and microcapsules, respectively. While the extract reduced the neutrophil content by 58%, there was no significant difference compared to the inflammation group.

3.4 Effect of EO and MO on the expression of IL-1β, IL 6, and TNF-α in model carrageenan-induced paw edema

The effects of *L. graveolens* extract and microcapsules on serum markers (IL-1β, IL-6, and TNF-α) were investigated in carrageenan-treated mice. As shown in Figure 4, carrageenan increased the serum levels of all three cytokines, with a significant difference ($p < 0.001$) compared to healthy mice. On the contrary, oregano extract and microcapsules orally administrated significantly inhibited the increase in serum levels of IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), and TNF-α ($p < 0.001$ and $p < 0.01$, respectively).

Figure 4. Effect of oregano extract and microcapsules on the levels of IL-1β, IL-6, and TNF-α in serum of mice with carrageenan-induced paw edema. All values were given as mean ± EEM.★ with a $p<0.05$, ★★ $p<0.01$ and ★★★ $p<0.001$ vs Naive; + with a $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ vs. Carrageenan group.

The treatment´s effect on cytokines in the plantar area was also investigated in carrageenan-treated mice. As shown in Figure 5, carrageenan increased IL-1β, IL-6, and TNF-α levels in the plantar area, with a significant difference ($p <0.001$) compared to healthy mice, indicating inflammation induction. In contrast, oral administration of oregano extract and microcapsules significantly inhibited the increase in cytokine levels ($p<0.05$), IL-1β, IL-6, and TNF-α.

Figure 5. Effect of oregano extract and microcapsules on the levels of IL-1β, IL-6, and TNF-α in plantar area of mice with carrageenan-induced paw edema. All values were given as mean ± EEM. With a ★ $p<0.05$, ★★ $p<0.01$ and ★★★ $p<0.001$ vs Naive; + with a $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ vs. Carrageenan group.

3.5 Effects of EO and MO on total and differential leukocytes and IgE total induced by ovalbumin in blood

Mice exposed to ovalbumin showed a significant increase in the number of inflammatory cells (leukocytes and lymphocytes) in blood compared to unexposed mice (Table 3). However, treatment with oregano extract, oregano microcapsules, or dexamethasone significantly inhibited ovalbumin-induced inflammatory cell infiltration. A feature of airway inflammation is the increased secretion of IgE into the blood.

As shown in Table 3, the amount of IgE was significantly increased in the ovalbumin-sensitized group compared with the untreated group, implying that the induction of inflammation was successful in this study. As a result, the secretion of IgE was significantly decreased by oregano extract and oregano microcapsules. These results demonstrate that oregano has a preventive effect in ovalbumin-induced mice by suppressing IgE and reducing airway infiltration.

Table 3. Immunoglobulin E total serum, differential white blood cell count and platelets in mice with ovalbumin-induced inflammation.

Group	IgE total (UI/mL)	Leukocytes	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
Naive	<10	1.62 ± 0.50	0.05 ± 0.03	1.31 ±0.03	0.03 ± 0.01	0.01 ± 0.01	0.33 ± 0.09	471 ± 130
Ovoalbumin	34.7 ± 4.7*	9.28 ±1.69*	0.31 ±0.05	5.39 ± 1.3*	0.45 ± 0.18	0.05 ± 0.01	0.61 ± 0.07	543 ± 79
Dexamethasone (2 mg/kg)	<10^{+}	4.32 ± 1.10^{+}	0.61 ± 0.13	2.71 ± 0.32^{+}	1.08 ± 0.35	0.02 ±0.01	0.75 ± 0.27	104 ± 20^{+}
Extract (560 mg/kg)	21.6 ±7.0^{+}	4.68 ± 0.76^{+}	0.28 ± 0.02	3.63 ± 0.94^{+}	0.07 ± 0.01	0.01 ± 0.00	0.58 ± 0.04	863 ± 90^{+}

Microcapsules (560 mg/kg)	17.0 ± 5.2^{+}	5.61 ± 1.55^{+}	0.27 ± 0.09	3.82 ± 0.38^{+}	0.07 ± 0.02	0.01 ± 0.00	0.29 ±0.07	649 ± 65

Values are expressed in (10^3/ mm^3). ⋆ Significant difference between two groups (Naive vs Ovalbumin; $p<0.5$).
$^{+}$ Significant difference regarding the Ovalbumin group ($p<0.05$).

3.6 Oregano extract and oregano microcapsules effect on IL-1β, IL-6, and TNF-α expression in lung airway inflammation induced by ovoalbumin

The levels of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, in the lung tissue of ovalbumin-stimulated ICR mice were examined. Cytokine levels were significantly higher than those in the unstimulated group (Figure 6). However, the oregano extract-treated group showed significantly reduced IL-1β, IL-6, and TNF-α levels.

Figure 6. Effect of oregano extract and microcapsules on IL-1β, IL-6, and TNF- α levels in airway inflammation model. All values were given as mean ± EEM.★ with a $p<0.05$, ★★ $p<0.01$ and ★★★ $p<0.001$ vs. Naive; + with a <0.05, ++ $p<0.01$ and +++ $p<0.001$ vs Ovalbumin-challenged group.

4. Discussion

This is the first study using oregano extract with an edema paw model. However, there are studies on some natural compounds, such as the study by Laavola et al. (2017), where they evaluated the lignan compound nortrachelogenin at a 100 mg/kg dose with a 53% effect. In another study by Rahmawati et al. (2022), an aqueous extract of *Prasiola japonica* at a 100 mg/kg dose had a 50% effect. The results of the microcapsules are also promising compared with other studies like Wang et al. (2015), where they found a 73% effect with glycyrrhizin

at 160 mg/kg, and Alblihed (2020), who showed 30% with astragalin at 75 mg/kg. Also, those studies evaluated chemical standards, while our samples (oregano extracts) are a mixture of several compounds. However, their administration method was intraperitoneal, indicating a more significant effect than ours since our oregano extracts were administered orally.

On the other hand, the quantification of inflammatory cells in the paw edema model showed that, despite using the same doses, there is a significant difference between the free extract and the microencapsulated extract, with the free extract being better. According to previous studies carried out on cell lines, this can be attributed to the excipient (maltodextrin) helping cell growth, so the reduction of inflammatory cells did not have the same efficiency. The anti-inflammatory response of oregano extracts and microcapsules, according to the content of inflammatory cells, showed behavior similar to that found in other studies and is mainly attributed to the action of flavonoids. Furtado et al. (2016), evaluated an aqueous extract of *Ipomoea asarifolia* leaves in an inflammation model with carrageenan that presented a reduction in leukocyte migration of up to 83%, attributing the effect mainly to phenolic compounds such as rutin, chlorogenic acid and caffeic acid. In another study, an extract of malacca leaves (*Phyllanthus emblica*), inhibited the accumulation of leukocytes, probably influenced by the presence of flavonoids (Asmilia et al., 2020).

Furthermore, other plant extracts such as *Morus alba* L. and *Morus mesozygia* Stapf (Moraceae) were evaluated with a reduction of leukocytes of up to 65%, attributing the effect to flavonoid compounds (Ariyo et al., 2020; Oliveira et al., 2016).

In addition, oregano treatments showed an anti-inflammatory effect on the inhibition of proinflammatory cytokines in both serum samples and paw tissue, which agreed with the results of other authors. For example, Gul et al. (2023), evaluated an aqueous extract of

Cassia with high quercetin content on an inflammatory arthritis model, reporting a decrease in the levels of IL-1β and IL-6. In a different study, Pinho-Ribeiro et al. (2015), reported the inhibition of cytokine production (TNF-α, IL-1β, IL-6, and IL-10) and NK-kB activation with the administration of hesperidin methyl chalcone (HMC). Also, the reduction of TNF-α and IL-1β (30-50%) with dry extract of *M. albicans* was reported, attributing the effect to flavonoids such as rutin and quercetin (Lima et al., 2020).

Regarding the inhibition of inflammatory cells in ovalbumin-induced inflammation models, the results found are similar to other studies. Lee et al. (2020), evaluated *Rosa leavigata* extracts diminishing allergic inflammation by reducing inflammatory cells. The same behavior was observed in other studies where "osthole" coumarin and *Cassia occidentalis* L. extract were evaluated (Wang et al., 2017; Xu et al., 2018). Regarding the decrease in IgE, although dexamethasone had a greater effect than oregano treatments, very low platelet levels were observed compared to the control when using the drug since it acts as an immunosuppressant. On the contrary, oregano samples maintained normal platelet levels, so that they could be a good option for immunocompromised patients. Immunoglobulin E (IgE) reduction was also observed when evaluating other types of extracts and metabolites. Chung et al. (2015), reported a 20% reduction in IgE serum levels in mice that consumed kaempferol-3-O-rhamnoside. Furthermore, Wei et al. (2016), also reported the inhibition of specific IgE levels by 33% with the metabolite angelicin. Other plant extracts that have been evaluated in ovalbumin models and have found reductions in IgE are *Cassia occidentalis* L. and *Eriobotrya japonica* (Kim et al., 2020; Xu et al., 2018).

Finally, regarding the quantification of cytokines in the lung, the results are consistent with other research. In a study by (Bui et al., 2017), they administered *Bupleurum chinense* extract to ovalbumin-sensitized mice, attenuating IL-1 β, IL-4, IL-5, IL-6, TNF- α expression in lung

homogenate. Also, TNF-α was reduced by approximately 80% in lung tissue with kaempferol-3-O-rhamnosid (Chung et al., 2015). Another compound evaluated was epigallocatechin gallate, reducing IL-1β, IL-4, and IL-6 levels in nasal lavage fluid and nasal mucosa (Fu et al., 2017). IL-1β, IL-6, and TNF-α are considered general inflammation markers, and several authors attribute that the inhibition of these proinflammatory cytokines occurs through the inactivation of p65 subunit phosphorylation of the NF-kB protein and the degradation in the cytoplasm of the regulatory protein IkBa (Chen et al., 2018).

5. Conclusions

The present study demonstrated that oregano (*L. graveolens*) extracts and microcapsules showed an anti-inflammatory effect against carrageenan-induced paw edema and allergic asthma in ovalbumin-challenged mice. Oregano administration decreased paw inflammation, reduced leukocyte and proinflammatory cytokine levels in both models and attenuated IgE levels in the airway inflammation model. Therefore, the *L. graveolens* aqueous extract is a promising natural anti-inflammatory agent.

6. Acknowledgements

To CONAHCYT for the Scholarship # 633460.

7. Authors' contributions

M.J. Bernal-Millán: Conceptualization, Investigation, Methodology, Formal analysis, Writing (original draft). **J.B Heredia**: Conceptualization, Investigation, Writing (review and editing). **F.J. Flores-Murrieta:** Conceptualization, Investigation, Resources and Writing (review and editing). **M.C. Carrasco-Portugal:** Conceptualization, Investigation,

Methodology, Project administration, Resources, Supervision, Visualization, Writing (review and editing). **J.E. Roa-Coria:** Formal analysis and Methodology. **J.C. Huerta-Cruz:** Formal analysis and Methodology. **E.P. Gutiérrez-Grijalva:** Formal analysis and Visualization. **J. León-Félix:** Formal analysis and Visualization. **M.A. Angulo-Escalante:** Formal analysis and Visualization.

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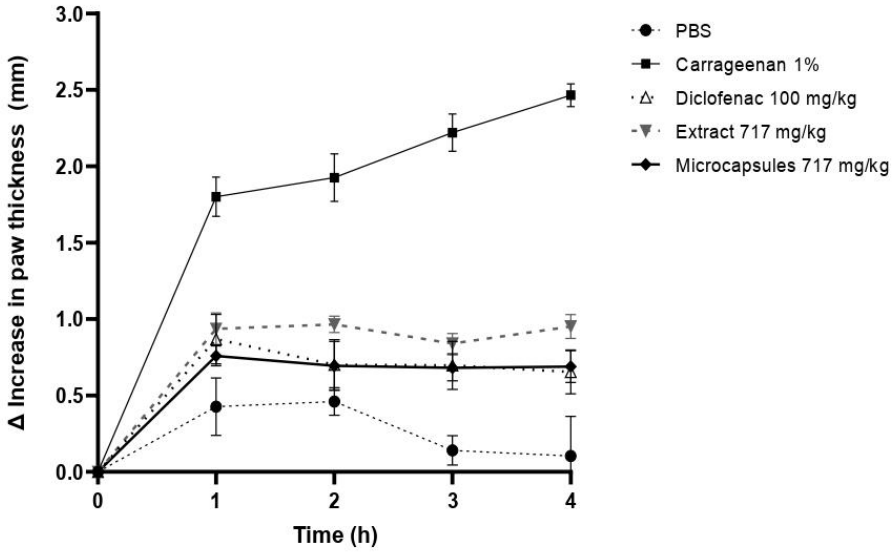

Figure 1. Time course of inflammation in a carrageenan-induced paw edema model.

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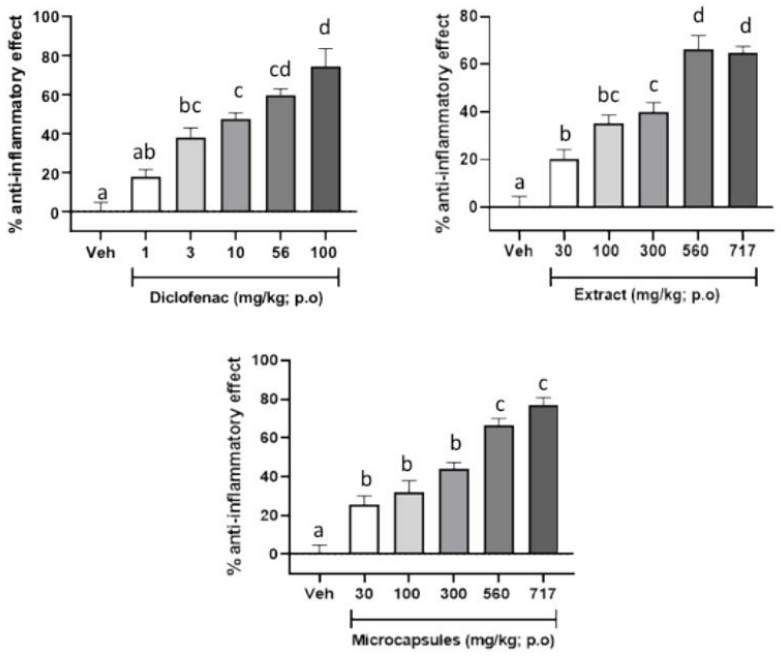

Figure 2. Anti-inflammatory effect of different doses of diclofenac, oregano extract, and oregano microcapsules on mice with carrageenan-induced paw edema. Significant difference between groups with different letters on the bars.

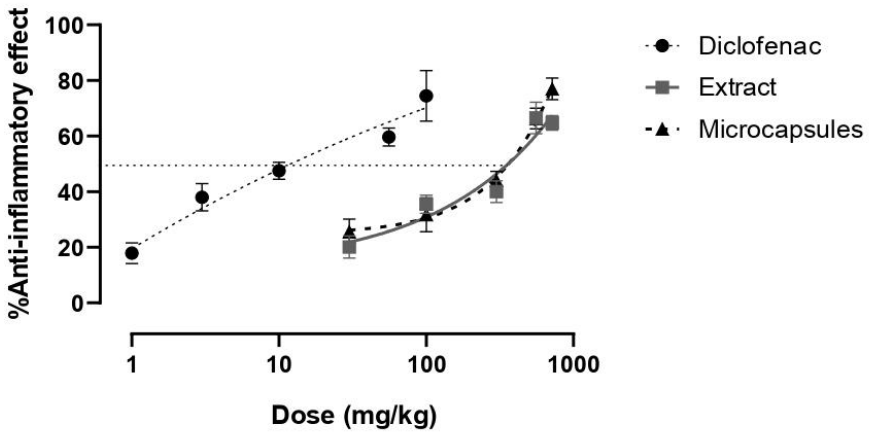

Figure 3. Dose-response curves of diclofenac, oregano extract, and microcapsules on their anti-inflammatory effect in a carrageenan-induced paw edema model.

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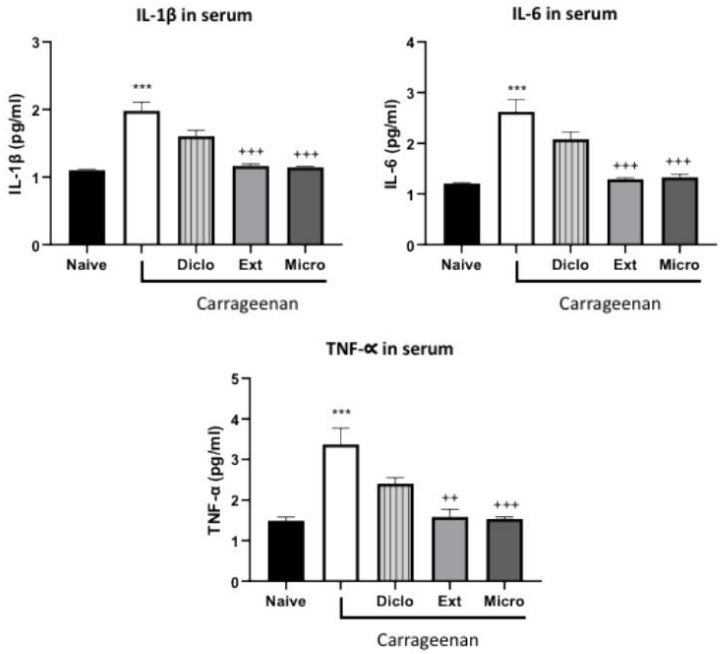

Figure 4. Effect of oregano extract and microcapsules on the levels of IL-1β, IL-6, and TNF-α in serum of mice with carrageenan-induced paw edema. All values were given as mean ± EEM.★ with a $p<0.05$, ★★ $p<0.01$ and ★★★ $p<0.001$ vs Naive; + with a $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ vs. Carrageenan group.

Figure_5 Click here to access/download;Figure;Figure_5.docx

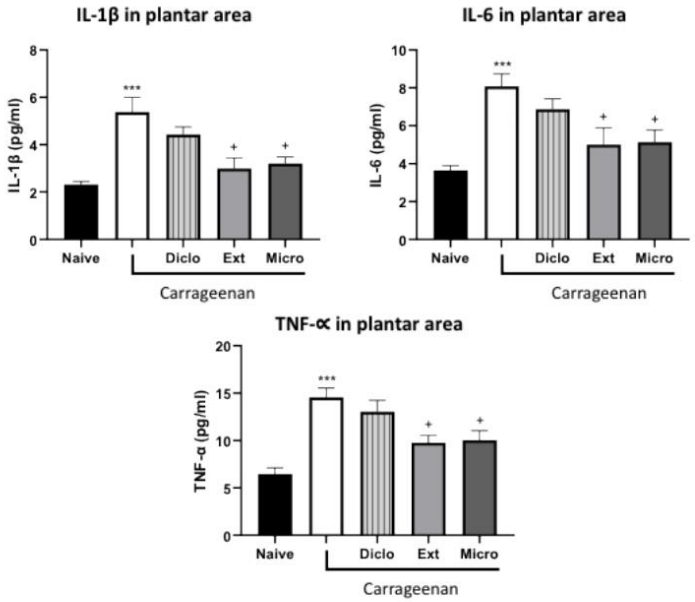

Figure 5. Effect of oregano extract and microcapsules on the levels of IL-1β, IL-6, and TNF-α in plantar area of mice with carrageenan-induced paw edema. All values were given as mean ± EEM. With a ★ $p<0.05$, ★★ $p<0.01$ and ★★★ $p<0.001$ vs Naive; + with a $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ vs. Carrageenan group.

Figure_6 Click here to access/download;Figure;Figure_6.docx

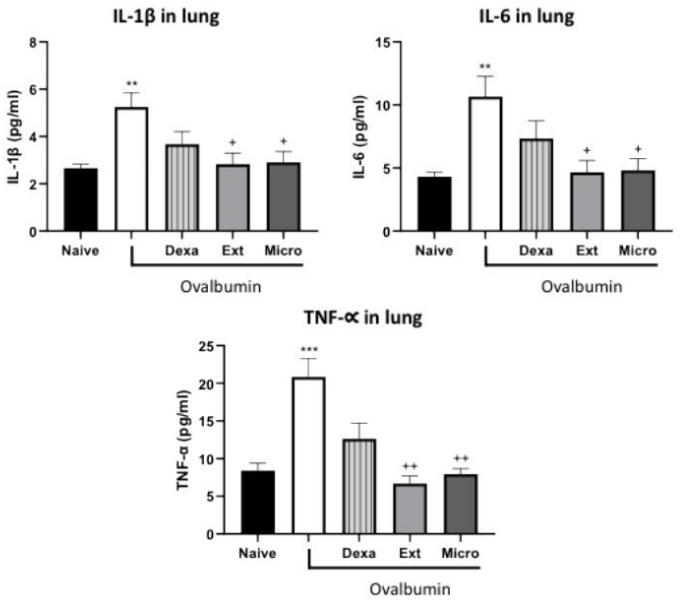

Figure 6. Effect of oregano extract and microcapsules on IL-1β, IL-6, and TNF- α levels in airway inflammation model. All values were given as mean ± EEM.★ with a $p<0.05$, ★★ $p<0.01$ and ★★★ $p<0.001$ vs. Naive; + with a <0.05, ++ $p<0.01$ and +++ $p<0.001$ vs Ovalbumin-challenged group.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

4. CONCLUSIONES GENERALES

Se encontró que tanto los métodos convencionales como las tecnologías emergentes de extracción de compuestos bioactivos como los flavonoides presentan altos rendimientos de extracción; sin embargo, el método de maceración con metanol y la extracción asistida por ultrasonido-DES mostraron los mejores resultados antioxidantes. También se realizó microencapsulación de flavonoides de orégano, logrando una encapsulación del 90.1%; diversas pruebas determinaron que las microcápsulas presentaban potencial antioxidante. Asimismo, se identificaron y cuantificaron 14 flavonoides, destacándose la naringenina y la floridzina, reportadas con alto potencial bioactivo.

Además, los extractos y microcápsulas de orégano (*L. graveolens*) mostraron un efecto antiinflamatorio contra el edema de la pata inducido por carragenina y el asma alérgica en ratones expuestos a ovoalbúmina, sin embargo, la microencapsulación no mejoro la potencia del efecto antiinflamatorio de manera significativa por lo que no es necesario realizar este proceso. La administración de orégano disminuyó la inflamación de la pata, redujo los niveles de leucocitos y citocinas proinflamatorias en ambos modelos y atenuó los niveles de IgE en el modelo de inflamación de las vías respiratorias. Por lo tanto, el extracto de *L. graveolens* es un agente antiinflamatorio natural prometedor contra enfermedades inflamatorias.

5. RECOMENDACIONES

- ✓ Evaluar los mecanismos de acción antiinflamatoria de los extractos de orégano.
- ✓ Utilizar estándares de los diferentes compuestos fitoquímicos de forma individual como referencia para atribuir propiedades de los compuestos del orégano con más certeza.
- ✓ Aislar y purificar compuestos de los extractos de orégano y realizar experimentos de potencial antiinflamatorio.
- ✓ Evaluar el potencial antinociceptivo de los compuestos individuales del orégano en modelos de ratones.