ESPECIES DE PLANTAS COLOMBIANAS CON POTENCIAL ANTIINFLAMATORIO SOBRE MODELOS DE INFLAMACIÓN TÓPICA Y ENFERMEDAD INFLAMATORIA INTESTINAL

COLOMBIAN PLANT SPECIES WITH ANTI-INFLAMMATORY POTENTIAL ON MODELS OF TOPICAL INFLAMMATION AND INFLAMMATORY BOWEL DISEASE

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Proyecto de grado presentado como requisito para optar el título de
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TABLA DE CONTENIDO

ÍNDICE DE FIGURAS.

ÍNDICE DE TABLAS.

ABBREVIATURAS

ABTS: 2,2′-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid)
$CO3^{\bullet -}$: carbonate
COX-2: Cyclooxygenase-2
DAI: Disease Activity Index
DPPH: 2,2-diphenyl-1-picrylhydrazyl
DCFH-DA: 2′,7′-dichlorofluorescein diacetate
DMEM: Dulbecco's modified eagle medium
DMSO: dimethyl sulfoxide
DSS: Dextran Sulfate Sodium
EDTA: ethylenediaminetetraacetic acid
ELISA: Enzyme-Linked ImmunoSorbent Assay
FBS: fetal bovine serum
FDA: Food and Drug Administration
FRAP: ferric reducing antioxidant power
GAE: gallic acid equivalents
GD: Golden Berry
HA: hydrogen atom
H_2O_2: hydrogen peroxide
H&E: hematoxylin and eosin
HTAB: Hexadecyltrimethylammonium bromide
IBD: inflammatory bowel disease
IC_{50}: inhibit concentration 50
IL-6: interleukins 6
IL-1β: interleukin 1β
IL-8: interleukin 8
IL-10: interleukin 10
IL-12: interleukin 12
IFN-b: interferon b
iNOS: nitric oxide synthase
IR: inhibition rates
LC_{50}: Lethal Concentration 50
LPS: lipopolysaccharide
MAPK: Mitogen-Activated Protein Kinases
(MCP)-1: monocyte chemotactic protein
MPO: Myeloperoxidase
MTT: Bromide of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium
NCDs: Chronic non-communicable diseases
$NaNO_2$: sodium nitrite

NO: nitric oxide
•NO_2: nitrogen dioxide
•O^{2-}: superoxide radical anion
OD: Optic Density
•OH: hydroxyl radicals
$ONOO^-$: peroxynitrite ion
ORAC-FL: oxygen radical absorbance capacity-fluorescein
PAS: Periodic Acid-Schiff
PBS: phosphate buffered saline
PGE2: prostaglandin E2
PKC: protein kinase C
PLA2: Phospholipase A2
QE: catechin equivalents
RNS: reactive nitrogen species
ROI: reactive oxygen intermediates
ROO: peroxyl radicals
ROS: reactive oxygen species
RT: room temperature
SARS-CoV2: severe acute respiratory syndrome coronavirus 2
SE: single electron
SEM: standard error of the mean
SI: Selectivity Index
SNP: sodium nitroprusside
TLR4: Toll-like receptor 4
TNF-α: tumor necrosis factor
TPA: 12-O-tetradecanoyl-phorbol-13-acetate
TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine
Trolox: 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid
WHO: World Health Organization
1400W: N′-[[3-(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride

RESUMEN

Las enfermedades crónicas no transmisibles (ECNT) son la principal causa de morbilidad y mortalidad en la población mundial, representando el 70% de todas las muertes en el mundo. La Organización Mundial de la Salud (OMS) estima que más de 14 millones de personas entre 30 y 70 años mueren anualmente en el mundo por una enfermedad crónica y el 85% de estas personas provienen de un país en desarrollo. La aparición de estas enfermedades está asociada a factores de riesgo modificables relacionados con el estilo de vida, como la inactividad física, el consumo nocivo de alcohol, tabaco y una dieta poco saludable; los cuales pueden producir un daño celular considerable en los tejidos diana y desencadenar un desequilibrio redox que produce un estado de "estrés oxidativo", a menudo acompañado o relacionado con un proceso inflamatorio crónico, el cual ha sido reconocido como un mecanismo patofisiológico subyacente en la aparición de muchas ECNT. En este sentido, la supresión de la respuesta inflamatoria en enfermedades crónicas puede afectar beneficiosamente el resultado de la misma. Colombia cuenta con una combinación única de atributos geográficos y topográficos que le han permitido contar con una extraordinaria diversidad de flora, siendo catalogado como uno de los países más megadiversos del mundo. Tradicionalmente un alto porcentaje de la población colombiana ha empleado especies vegetales con propiedades medicinales como alternativas terapéuticas para tratar diversas enfermedades que cursan con inflamación. Sin embargo, muchas de estas especies han sido poco o nada estudiadas por nuestra comunidad científica. Este proyecto contribuyó al conocimiento del potencial anti-inflamatorio de algunas especies vegetales colombianas, evaluando inicialmente el efecto de cincuenta y uno (51) extractos vegetales, sobre la liberación del mediador inflamatorio óxido nítrico (NO•) en macrófagos RAW 264.7. Los resultados permitieron identificar el potencial anti-inflamatorio de los extractos etanólicos de las semillas de *Ambrosia peruviana,* las hojas de *Mammea americana,* los cálices de *Physalis angulata,* y el fruto de *Physalis peruviana*; los cuales además de inhibir la producción de NO•, también disminuyeron significativamente la liberación de TNF-α, IL-1β, IL-6 y PGE-2 en macrófagos RAW 264.7 activados. Estos resultados se consideran prometedores teniendo en cuenta que se trata de biomarcadores de inflamación importantes en los procesos inflamatorios asociados a las ECNT. Con miras a darle continuidad al estudio de estas promisorias especies y obtener resultados a nivel in *vivo,* se evaluó el efecto anti-inflamatorio del extracto total etanólico de las hojas de *Mammea americana* y los cálices de *Physalis angulata,* en el modelo de edema auricular inducido por TPA. Adicionalmente, se evaluó el potencial anti-inflamatorio del extracto etanólico total de las flores de *Cordia alba,* en el mismo modelo, siendo estos resultados el primer reporte del potencial anti-inflamatorio de las flores de esta especie. Los resultados mostraron que los tres extractos lograron disminuir significativamente el edema producido por el agente irritante TPA y disminuyeron la infiltración de neutrófilos en el tejido. Por otro lado, el extracto del fruto de *Physalis peruviana,* fue evaluado en el modelo de enfermedad inflamatoria intestinal inducida por dextran sulfato de sodio (DSS) en ratones BALB/c, los resultados mostraron que una dieta suplementada con extracto del fruto de *Physalis peruviana* atenuó los síntomas de la colitis aguda inducida por el DSS, reduciendo el sangrado, la pérdida de peso, la diarrea y el acortamiento del colon. Adicionalmente, este extracto inhibió la actividad de la enzima MPO y disminuyó la producción de IL-6, IL-1β, IL-10, TNF-α y EROs en el tejido colónico. Los resultados de este proyecto demuestran el efecto anti-inflamatorio de los extractos obtenidos de las hojas de *Mammea americana,* de los cálices de *Physalis angulata,* las flores de *Cordia*

alba y los frutos de *Physalis peruviana*, convirtiéndose en la base de posteriores estudios encaminados al desarrollo de fitopreparados y alimentos funcionales con utilidad para la prevención y/o el tratamiento de enfermedades crónicas no transmisibles.

ABSTRACT

Chronic non-communicable diseases (NCDs) are the leading cause of morbidity and mortality in the world population, accounting for 70% of all deaths worldwide. According to recent estimates by the World Health Organization (WHO), more than 14 million people between the ages of 30 and 70 die annually worldwide from a chronic disease and 85% of these people come from a developing country. The appearance of these diseases are associated with modifiable risk factors related to lifestyle, such as physical inactivity, harmful consumption of alcohol, tobacco and an unhealthy diet; which can produce considerable cellular damage in the target tissues and trigger a redox imbalance that produces a state of "oxidative stress", often accompanied or related to a chronic inflammatory process, which has been recognized as an underlying pathophysiological mechanism in the appearance of many NCDs. In this sense, the suppression of the inflammatory response in chronic diseases can beneficially affect the result of it. Colombia has a unique combination of geographic and topographic attributes that have allowed it to have an extraordinary diversity of flora, being listed as one of the most megadiverse countries in the world. Traditionally, a high percentage of the Colombian population has used plant species with medicinal properties as therapeutic alternatives to treat various diseases that cause inflammation. However, many of these species have been little or nothing studied by our scientific community. This project contributed to the knowledge of the anti-inflammatory potential of some Colombian plant species, initially evaluating the effect of fifty-one (51) plant extracts on the release of the inflammatory mediator nitric oxide (NO•) in RAW 264.7 macrophages. The results allowed to identify the anti-inflammatory potential of the ethanolic extract of the seeds of *Ambrosia peruviana*, the leaves of *Mammea americana*, the calyces of *Physalis angulata*, and the fruit of *Physalis peruviana*, that in addition to inhibiting NO • production, they also significantly decreased the production of TNF-α, IL-1β, IL-6 and PGE-2, in RAW 264.7 macrophages. These results are considered highly promising considering that these are important biomarkers of inflammation in the inflammatory processes associated with NCDs. In order to give continuity to the study of these species identified as promising and to obtain results at the *in vivo* level, the anti-inflammatory effect of the total ethanolic extract of the leaves of *Mammea americana* and the calyxes of *Physalis angulata* was evaluated in the TPA-induced mouse ear edema model. Additionally, the anti-inflammatory potential of the total ethanolic extract of Cordia alba flowers was evaluated, in the same model, being these results the first report of the anti-inflammatory potential of the flowers of this species. The results showed that three extracts were able to significantly reduce the inflammation produced by the irritant agent TPA and decreased the infiltration of neutrophils into the tissue. On the other hand, the extract from the fruit of the *Physalis peruviana* species was evaluated in the DSS-induced inflammatory bowel disease model in BALB/c mice, the result shows that a diet supplemented with *Physalis peruviana* fruit extract attenuated the pathological symptoms of dextran sulfate sodium (DSS)-induced acute colitis, reducing the bleeding, weight loss, diarrhea and shortening of the colon. Additionally, this extract inhibited the activity of the MPO enzyme and decreased the production of IL-6, IL-1β, IL-10, TNF-α and ROS in the colon tissue. The results of this project show the anti-inflammatory effect of the leaves of *Mammea americana*, the calyxes of *Physalis angulate*, the flowers of *Cordia alba* and the fruits of *Physalis peruviana*, becoming the basis for subsequent studies aimed at the development of phytopreparations and functional foods useful for prevention and/or the treatment of chronic non-communicable diseases.

DESCRIPCIÓN GENERAL DE LA TESIS DOCTORAL

Las enfermedades crónicas no transmisibles (ECNT), son enfermedades no infecciosas y de larga duración (más de tres o seis meses). En general, se desarrollan de manera lenta y asintomática, pero conducen a complicaciones devastadoras, lo que resulta en una muerte prematura y una mala calidad de vida. Las ECNT son la principal causa de mortalidad a nivel mundial. Se estima que el 70% de todas las muertes en el mundo son atribuibles a enfermedades crónicas no transmisibles (Alwan et al., 2010; Holt et al., 2018; Meetoo, 2008; Ruby et al., 2015; Senthilkumar & Kim, 2013). Numerosas revisiones sistemáticas han demostrado asociación entre biomarcadores inflamatorios, tales como factores de transcripción (NF-κB y STAT3), citoquinas inflamatorias (TNF-α, IL-1, IL-6 y IL-8), enzimas proinflamatorias (COX-2, 5-LOX, 12-LOX y metaloproteinasas de matriz), quemoquinas, proteína C reactiva (CRP), entre otros; con la aparición y progresión de varias ECNT (Prasad et al., 2012). Adicionalmente, la respuesta inflamatoria crónica que se presenta en estas patologías contribuye a la producción exacerbada de ROS y RNS, desencadenando un estado de estrés oxidativo, el cual puede agravar la patología o contribuir al desarrollo de otras (Lee et al., 2015; Liguori et al., 2018; Virág et al., 2019). Por tanto, la identificación de sustancias o compuestos capaces de disminuir la producción de mediadores inflamatorios y mitigar el estrés oxidativo sería una estrategia útil y eficiente para la prevención y/o tratamiento de ECNT.

En este apartado se presenta la concepción y pertinencia de este proyecto de tesis, incluyendo su hipótesis y objetivo principal. Esta investigación se estudiaron cincuenta y uno (51) especies vegetales colombianas capaces de inhibir la producción de mediadores inflamatorios, sirviendo así de punto de partida para la elaboración de nuevos tratamientos capaces de contrarrestar la inflamación que caracteriza a las ECNT. Estas cincuenta y uno especies fueron seleccionadas en dos etapas: en la primera etapa se seleccionaron 41 especies vegetales usadas ampliamente en la medicina popular por sus propiedades medicinales y en la segunda etapa se seleccionaron 10 especies comestibles, cuyos frutos hacen parte de la dieta colombiana y que su consumo regular en la dieta se asociado con beneficios para la salud.

Etapa I

A partir de las 41 especies vegetales seleccionadas en la primera etapa se obtuvieron 47 extractos, los cuales se evaluaron en dos bloques y cuyos resultados generaron un total de tres productos de nuevo conocimiento, los cuales se encuentran consignados en el Capitulo I de esta Tesis. El primer bloque contaba con 37 extractos, los cuales fueron seleccionados de acuerdo a la información recolectada a partir de una revisión bibliográfica en diferentes bases de datos y encuestas realizadas a la comunidad sobre las propiedades medicinales de estas plantas. En el producto I de esta tesis se encuentran los resultados obtenidos de la evaluación

del potencial anti-inflamatorio *in vitro* de estos 37 extractos, identificándose el extracto obtenido a partir de las hojas de *Mammea americana* como promisorio, y por lo tanto se evaluó su capacidad para inhibir la formación de edema en un modelo *in vivo* de edema auricular inducido por TPA. Los resultados del potencial anti-inflamatorio *in vivo* de esta especie vegetal se encuentran descritos en el producto II de este trabajo.

El segundo bloque esta conformado por 10 extractos que fueron seleccionados contemplando la información recolectada a partir de la revisión bibliográfica en diferentes bases de datos y encuestas realizadas a la comunidad sobre las propiedades medicinales de estas plantas, así como considerando los resultados previos obtenidos en trabajos de grado realizados al interior del grupo de investigación Evaluación Biológica de Sustancias Promisorias. Los resultados obtenidos de la evaluación *in vitro* del potencial anti-inflamatorio de los 10 extractos que conforman este II bloque se encuentran en el producto III de esta tesis doctoral. Teniendo en cuenta estos resultados, así como los criterios de inclusión de estos 10 extractos, se seleccionaron los extractos obtenidos de los cálices de *Physalis angulata* y las flores de *Cordia alba* para ser evaluados en el modelo *in vivo* de edema auricular inducido por TPA. Los resultados del potencial anti-inflamatorio *in vivo* de estas especies vegetales se encuentran consignados en los productos III y IV, resaltándose el importante efecto anti-inflamatorio *in vivo* presentado por ambos extractos.

Etapa II

En la segunda etapa de este proyecto se prepararon los extractos etanólicos totales de 10 frutos comestibles y se evaluó su potencial antioxidante y anti-inflamatorio, estos resultados se encuentran descritos en el Capitulo 2, productos V y VI. En el producto V se encuentran los resultados relacionados el potencial antioxidante de estos extractos, el cual fue evaluado empleando los métodos de DPPH, ABTS, FRAP, ORAC y DCFH-DA. Los resultados presentados en este producto destacan el potencial antioxidante de los extractos de pitaya amarilla, curuba y uchuva. Mientras que en el producto VI se describen los resultados relacionados con la actividad anti-inflamatoria *in vitro* de estos 10 extractos comestibles, siendo las frutas más activas como anti-inflamatorias la gulupa, pitaya amarilla, tomate de árbol y uchuva. Si bien todas las frutas mencionadas anteriormente resultaron ser útiles para disminuir la concentración de uno o varios mediadores, el extracto más activo fue el de la uchuva, por lo que fue seleccionado para determinar su potencial antiinflamatorio *in vivo* en un modelo de enfermedad inflamatoria intestinal inducido por DSS, con resultados notables, reduciendo de manera significativa la inflamación a nivel intestinal.

Finalmente, en el Capítulo 3 se presenta una discusión general sobre los principales aportes de esta tesis en relación al potencial anti-inflamatorio de las hojas de *Mammea americana*, los cálices de *Physalis angulata*, las flores de *Cordia alba* y los frutos de *Physalis peruviana*. Además de una sección de conclusiones finales y desafíos y perspectivas futuras.

Concepción y pertinencia del estudio

La prevención y el tratamiento temprano de las ECNT es un desafío mundial al que la Organización Mundial de la Salud (OMS) asigna una alta prioridad. Las ECNT generalmente tienen una etapa prodrómica prolongada que a menudo demora muchos años en desarrollarse, como resultado, el potencial de desarrollar una ECNT aumenta a medida que la población envejece. Por lo tanto, una estrategia preventiva temprana, es un enfoque ideal para ralentizar o incluso detener los procesos degenerativos que se desarrollan tanto por el envejecimiento como por los hábitos relacionados con el estilo de vida (Seyedsadjadi & Grant, 2021).

En la búsqueda de nuevas alternativas para prevenir la aparición de ECNT, los productos naturales han mostrado jugar un papel importante, lo cual no resulta extraño, ya que los productos naturales han contribuido significativamente al desarrollo de la medicina moderna, siendo estos una fuente fundamental en el descubrimiento de fármacos como antibióticos, agentes anticancerígenos, analgésicos y compuestos anti-inflamatorios (Fabricant & Farnsworth, 2001; Kingston, 2011; Sen & Samanta, 2014). De hecho, aproximadamente la mitad de los fármacos aprobados por la FDA entre 1980 y 2010, fueron productos naturales o derivados sintéticos relacionados con productos naturales (Chen et al., 2015; Ovadje et al., 2015). En este sentido, el estudio de plantas medicinales sigue siendo un área importante en la búsqueda de compuestos que sean de utilidad tanto en la prevención como en el tratamiento de ECNT, ya que especies vegetales con fitoquímicos capaces de inhibir la inflamación y el estrés oxidativo, podría ser una alternativa prometedora tanto para la prevención como para el tratamiento de las ECNT.

Colombia cuenta con una enorme diversidad biológica, con una gran variedad de especies vegetales que representan un inmenso potencial en términos de compuestos anti-inflamatorios y antioxidantes de origen natural. De hecho, tradicionalmente un alto porcentaje de la población colombiana ha empleado especies vegetales con propiedades medicinales como alternativas terapéuticas para tratar diversas enfermedades que cursan con inflamación. Sin embargo, muchas de estas especies han sido poco o nada estudiadas por nuestra comunidad científica. Se estima que Colombia alberga entre 45 a 50 mil especies de plantas, lastimosamente para un elevado porcentaje de estas especies se cuenta con un pobre conocimiento, lo que ha evitado aprovechar los beneficios que puede aportar esta flora en la alimentación, medicina e industria, mientras se está deteriorando su valor estético natural y su aporte al equilibrio global (Tobasura, 2006).

Una vía alternativa para aprovechar en mayor medida estos recursos biológicos es la práctica de la bioprospección, la cual implica la interacción entre diferentes tipos de conocimiento, especialmente aquel conocimiento ancestral con los que cuentan las comunidades locales e indígenas de los países sudamericanos y el conocimiento científico o tecnológico aportado

por los sectores académicos e investigativos y las actividades comerciales propias del sector industrial especialmente la industria farmacéutica. Para la industria farmacéutica, Colombia tiene un enorme atractivo para emprender prácticas de bioprospección, debido a su gran biodiversidad, que representa una enorme fuente de compuestos químicos y biológicos novedosos con gran potencial de comercialización; pero desafortunadamente se cuenta con muy pocos estudios que contribuyan a cuantificar este potencial (Duarte & Velho, 2010). Con el fin de contribuir a la bioprospección de nuestros recursos naturales y en concordancia con la permanente necesidad de identificar nuevos sustancias o compuestos con propiedades anti-inflamatorias y antioxidantes, en la ejecución de este trabajo se evaluó el potencial anti-inflamatorio de 51 especies vegetales colombianas.

Hipótesis:

"Es posible identificar en la flora colombiana especies vegetales potencialmente útiles en la prevención o tratamiento de enfermedades crónicas no transmisibles asociadas a procesos inflamatorios y estrés oxidativo"

Objetivos:

Para probar esta hipótesis, el objetivo general de esta Tesis Doctoral fue: "Realizar un tamizaje farmacológico que permita identificar especies de la flora colombiana con potencial actividad anti-inflamatoria y antioxidante"

Para cumplir con este objetivo general, se plantearon en esta Tesis Doctoral los siguientes objetivos específicos:

- Evaluar el potencial anti-inflamatorio de especies vegetales utilizadas en la medicina popular de Colombia a través de un esquema biodirigido *in vitro*, utilizando el mediador óxido nítrico.

- Determinar el efecto de las especies vegetales identificadas con potencial actividad anti-inflamatoria sobre los biomarcadores de inflamación IL-6, IL-1β, TNF-α y PGE2 en macrófagos RAW 264,7.

- Determinar el potencial antioxidante de las especies vegetales identificadas con potencial actividad anti-inflamatoria evaluando el efecto sobre la captación de radicales libres y sobre los niveles de EROs.

- Evaluar el efecto anti-inflamatorio *in vivo* de las especies vegetales identificadas como promisorias, determinando el efecto sobre los mediadores IL-6, IL-1β, TNF-α y PGE2.

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INTRODUCCIÓN

Enfermedades crónicas no transmisibles

Las enfermedades crónicas no transmisibles (ECNT), hace referencia a aquellas afecciones de tipo no infeccioso, que explican la mayoría de las enfermedades relacionadas con el envejecimiento, dentro de estas se incluyen las enfermedades cardiovasculares, neurodegenerativas, cáncer, diabetes mellitus, enfermedad renal crónica, enfermedad inflamatoria intestinal, entre otras. La mayoría de estas enfermedades pueden ser tratadas más no curadas, estas se desarrollan lentamente, pero llevan a complicaciones devastadoras, lo que resulta en muerte prematura y mala calidad de vida (Meetoo, 2008; Pareja-Galeano et al., 2015; Seyedsadjadi & Grant, 2021).

Como informó la Organización Mundial de la Salud (OMS), las enfermedades crónicas no transmisibles (ECNT) han aumentado durante el último siglo y representan la principal causa de muerte y discapacidad para la población en general, independientemente de la edad, la región o el sexo. De los 56,4 millones de muertes que se reportaron a nivel mundial durante el año 2015, el 70% correspondientes a 39,5 millones, se debieron a ECNT y se estima que más de 14 millones de personas entre 30 y 70 años mueren anualmente en todo el mundo por una enfermedad crónica y el 85% de estas personas provienen de un país en desarrollo. Este es un resultado costoso en términos de morbilidad personal, el rápido incremento en la incidencia y prevalencia de las ECNT imponen grandes costos humanos, sociales y económicos; ejerciendo una presión adicional sobre los sistemas de asistencia sanitaria y social, de hecho, se estima que las ECNT por sí solas absorberán más de 60 billones de dólares de la economía mundial entre el 2011 y el 2030 (Divajeva et al., 2014; Kimokoti & Millen, 2016; Pareja-Galeano et al., 2015; Seyedsadjadi & Grant, 2021)

Inflamación y estrés oxidativo: inductores de la aparición de enfermedades crónicas no transmisibles.

Las ECNT son enfermedades de progresión generalmente lenta y los síntomas clínicos solo se hacen evidentes después de que se ha producido un daño celular considerable en los tejidos diana. La investigación a la fecha, respalda el argumento de que este daño en los tejidos está influenciado en gran medida por cambios bioquímicos asociados con un desequilibrio redox que produce un estado de "estrés oxidativo crónico", a menudo acompañado o relacionado con un proceso inflamatorio conocido como para-inflamación o inflamación en un bajo grado. El estrés oxidativo y la para-inflamación pueden ser el resultado de hábitos relacionados con el estilo de vida como: consumo de tabaco, alcohol, sedentarismo y una dieta insaludable; los cuales pueden causar el mal funcionamiento de los tejidos y por ende la aparición de ECNT (Azevedo et al., 2014; Meetoo, 2008).

La para-inflamación puede ser causada por tejidos que se encuentren estresados o que no se encuentren funcionando bien. En condiciones basales, los tejidos se mantienen en un estado homeostático, mientras que, en condiciones nocivas, los tejidos sufren estrés y pueden funcionar mal. Los tejidos que sufren estrés pueden producir cantidades excesivas de especies reactivas de oxígeno (EROs), causando la activación de macrófagos residentes en el tejido o de aquellos que han sido reclutados recientemente, los cuales tratan de restablecer el buen funcionamiento del tejido. Sin embargo, si los estímulos que causan el mal funcionamiento del tejido persisten, los macrófagos activados, también liberan grandes cantidades de EROs y especies reactiva de nitrógeno (ERN). La exposición crónica a estas sustancias fuertemente oxidantes (EROs y ERN), pueden dañar aún más los tejidos. Es así como el estrés oxidativo y la inflamación se potencian entre si, causando un daño celular progresivo que se acumula con el tiempo. Este daño facilita la remodelación tisular que eventualmente da como resultado la aparición de ECNT (Medzhitov, 2008; Seyedsadjadi & Grant, 2021).

Las EROs y ERN contribuyen al deterioro de los tejidos al causar daños graves a las estructuras celulares, incluidos los lípidos, las proteínas y las moléculas de ADN; alterando sus funciones y provocando daños fundamentales en toda la célula. Los lípidos oxidados pueden formar diversos aldehídos citotóxicos, mientras que en las proteínas la oxidación de grupos tioles en residuos de cisteína, la nitración del anillo fenólico en residuos de tirosina o la adición de un grupo carbonilo como cetona o aldehído a la cadena lateral de aminoácidos como prolina, treonina, lisina y arginina pueden ocasionar perdida de la función de las proteínas, acelerando su degradación. En el ADN la oxidación de las bases nitrogenadas, por parte de estos radicales puede ocasionar mutaciones y en consecuencia la aparición de carcinogénesis. También pueden abstraer átomos de hidrógeno de la cadena principal de azúcar y fosfato del ADN, promoviendo la generación de roturas de cadenas de ADN. Estas modificaciones moleculares, son las responsables de alterar las funciones biológicas de las células, dando lugar a la aparición de diversas patologías de tipo crónico (Bogdan, 2015; Rahman et al., 2006; Seyedsadjadi & Grant, 2021).

Por otro lado, durante la respuesta inflamatoria, los macrófagos activados además de liberar EROs y ERN, activan diversos factores de transcripción como NF-κB y AP-1, estos factores pueden activar el sistema inmunológico innato con el posterior reclutamiento y acumulación de células inflamatorias como macrófagos, leucocitos y neutrófilos en el tejido dañado. Estas células inflamatorias pueden producir diversos mediadores inflamatorios como el ácido araquidónico, quimiocinas y citocinas pro-inflamatorias como la interleuquina IL-1β, IL-6 y el factor de necrosis tumoral alfa (TNF-α), mediadores inflamatorios que a medida que las ECNT progresa, se incrementa su concentración (Medzhitov, 2008; Seyedsadjadi & Grant, 2021).

Fitoquímicos como alternativa para la prevención y el tratamiento de ECNT.

Las propiedades farmacológicas de especies vegetales se deben en parte a la presencia de fitoquímicos, estos son compuestos con características químicas y funcionales muy diversas, que se derivan del metabolismo secundario de plantas, bacterias, hongos e incluso animales (González-Esquinca & Castro-Moreno, 2017; Pérez-Urria Carril & Ávalos García, 2009). Estudios han demostrado que un gran número de fitoquímicos podrían ser útiles para prevenir o tratar numerosas enfermedades crónicas. Los fitoquímicos más destacados como anti-inflamatorios son los terpenos, alcaloides y los compuestos fenólicos, estos últimos además gozan de muy buena fama como antioxidantes. Hasta el momento miles de compuestos fitoquímicos han sido identificados, sin embargo, un alto porcentaje de ellos sigue siendo desconocido (Liu, 2013a).

Los terpenos se encuentran distribuidos ampliamente en la naturaleza. Son una clase grande y variada de hidrocarburos que son producidos por una gran variedad de plantas y por algunos animales. En las plantas funcionan como infoquímicos, atrayentes o repelentes, ya que son los responsables de la fragancia típica de muchas plantas. Se describe una amplia gama de propiedades biológicas para los terpenos, que incluye actividad anticancerígena, antimicrobianas, antifúngica, antiviral, antihiperglucémicas, antiparasitarias y anti-inflamatorias. Esta ultima actividad biológica ha sido evaluada tanto *in vitro* como en modelos *in vivo;* y como resultado de estos estudios se ha podido identificar el potencial anti-inflamatorio de varios compuestos terpénicos como α-pineno, d-limoneno, p-cimeno, linalol, γ-terpineno, borneol, β-cariofileno, y 1,8-cineol, avicina, oleandrina, excisanina, kamebakaurina, costunolida, partenolida, entre otros (Cho et al., 2017; Paduch et al., 2007).

Los alcaloides son un grupo diverso de compuestos que contienen nitrógeno y han sido reconocidos por sus efectos anticancerígenos, analgésicos, antimicrobianos, antiarrítmicos, antihistamínicos y anti-inflamatorios (Sobarzo-Sánchez 2014). Dentro de los alcaloides con propiedades anti-inflamatoria se encuentran la vallesamina, warifteine, theacrine, tetrandrina, scholaricine, picrinina, ligustrazina, fangchinoline, berberina, entre otros (Barbosa-Filho et al., 2006; Souto et al., 2011).

Los compuestos fenólicos son metabolitos secundarios de plantas caracterizados por la presencia de un anillo aromático en su estructura que poseen mínimo un grupo hidroxilo. Los compuestos fenólicos se pueden dividir según el número de anillos fenólicos y los enlaces que se unen a los anillos en: ácidos fenólicos, estilbenos, flavonoides, lignanos, taninos y cumarinas. Estos compuestos juegan un papel vital en la reproducción, metabolismo y crecimiento de las plantas. Actúan como mecanismos de defensa contra infecciones causadas por virus, hongos, parásitos y depredadores (Del Rio et al., 2013; Khoo et al., 2016; Kris-Etherton et al., 2002; Liu, 2013a, 2013b; Pandey & Rizvi, 2009; Pierson et al., 2012; Zanotti et al., 2015). Además de sus funciones en las plantas, compuestos fenólicos como resveratrol,

kaempferol, quercetina, naringenin, daidzein, epigalocatequina-3-galato, epigalocatequina, epicatequina-3-galato, epicatequina, curcumina, entre otros; son conocidos tradicionalmente por su actividad como antioxidante y anti-inflamatoria (Costa et al., 2012; De La Lastra & Villegas, 2005; Kunnumakkara et al., 2017; Pan et al., 2010; Rahman, 2008; Santangelo et al., 2007).

El papel de la dieta en la aparición de enfermedades crónicas no transmisibles

La nutrición subóptima ocupa el lugar más alto entre los factores de riesgo del estilo de vida que desencadenan las ECNT en todo el mundo, este tipo de enfermedades son impulsadas por cambios en la dieta. En las últimas décadas, como consecuencia de la globalización, la población en general ha adoptado estilos de vida occidentales, incluyendo patrones dietéticos insalubres, esta tendencia es conocida como "transición nutricional", caracterizada por una sustitución de la dieta tradicional rica en frutas, verduras y carbohidratos complejos; por una dieta rica en calorías derivadas de grasas animales, y carbohidratos simples. Estos cambios en los hábitos alimentarios y estilo de vida imprimen un aumento significativo en la prevalencia del sobrepeso y obesidad, lo cual es considerado como uno de los principales factores de riesgo de ECNT. A excepción de los países con una infraestructura menos desarrollada, esta transición está en marcha en casi todos los rincones del mundo. Dicha dieta, combinada con un bajo nivel de actividad física, consumo regular de tabaco y consumo de alcohol, prepara el escenario para el desarrollo de este tipo de enfermedades (Astrup et al., 2008; Kimokoti & Millen, 2016; Meetoo, 2008).

En reconocimiento al impacto de una dieta saludable, para prevenir la aparición de ECNT, la Organización Mundial de la Salud (OMS) ha emitido recomendaciones dietéticas de expertos encaminadas a reducir el riesgo de padecer estas enfermedades. Estas recomendaciones consisten en un alto consumo de verduras, frutas, granos enteros, mariscos, legumbres y nueces; consumo moderado de alcohol y productos lácteos bajos en grasa, y por último un bajo consumo de carnes rojas, alimentos procesados; bebidas azucaradas y granos refinados (Kimokoti & Millen, 2016). La importancia de la dieta con relación al riesgo de padecer ECNT, está fundamentada en los estudios que demuestran que muchos de los fitoquímicos que se consumen a diario en la dieta, pueden modular el proceso inflamatorio crónico y el estrés oxidativo, los cuales han sido reconocidos como un mecanismo patofisiológico subyacente en la aparición de ECNT, como se mencionó anteriormente (Pan et al., 2010).

Fitoquímicos bioactivos en las frutas

Las frutas constituyen un grupo de alimentos de gran importancia en la nutrición humana, ya que son una fuente significativa de vitaminas, minerales y fibra dietética (Palafox-Carlos et al., 2011). El alto consumo de frutas en la dieta se ha asociado a una disminución en el riesgo de padecer enfermedades crónicas y degenerativas, mientras que una baja ingesta de las

mismas se encuentra entre los diez principales factores de riesgo implicados en la mortalidad global (Khoo et al., 2011; Slavin & Lloyd, 2012).

La asociación que existe entre el consumo de frutas y la disminución de los riesgos de padecer diversas ECNT, no solo ha sido atribuido a su alto contenido de vitaminas y minerales, sino a los fitoquímicos bioactivos que pueden producir, entre los cuales se encuentran compuestos fenólicos, flavonoides, carotenoides, ácidos grasos poliinsaturados; muchos de los cuales han demostrado su capacidad para modificar procesos inflamatorios crónicos modulando cascadas de señalización celular y por ende inhibiendo la síntesis y/o la acción de citocinas, quemoquinas o moléculas de adhesión (Ayala-Zavala et al., 2010; Heber, 2004; Prasad et al., 2012). Lo cual es consistente con los resultados obtenidos por los estudios realizados por Hebert, *et al* 2004; donde se encontró que constituyentes de las frutas como: fibra, vitaminas (A, C, D, E, B6), carotenoides, flavonas, flavonoles, flavonones, antocianidinas, isoflavonas, omega 3, entre otros, fueron categorizados como sustancias de la dieta con propiedades anti-inflamatorias; siendo estas sustancias catalogadas como los principales candidatos responsables de la actividad antioxidante, anti-inflamatoria, anticancerígena y neuroprotectora; asociada al consumo de frutas (Cavicchia et al., 2009; Heber, 2004; Shivappa et al., 2014).

La fibra consiste en material vegetal comestible, no digerible que incluye polisacáridos sin almidón y lignina (Chapman-Kiddell et al., 2010). El consumo de fibra está relacionado con el aumento de la excreción de ácidos biliares, estrógenos y procarcinógenos fecales. Además, promueve la absorción lenta de glucosa (mejorando la sensibilidad a la insulina), disminuye el colesterol sérico, inhibe la peroxidación lipídica y proporcionan propiedades anti-inflamatorias (Chiba et al., 2015). Se ha encontrado que la fibra fermentada por bacterias anaerobias del colon, da lugar a ácidos grasos de cadena corta (AGCC): acetato, propionato y butirato, este último compuesto reduce la inflamación de la mucosa al disminuir la actividad del Factor Nuclear-kappa-B (NF-κB) en las células del colon y aumentar la población de linfocitos T reguladores por inhibición directa de la enzima histona deacetilasa (HDAC) (Galvez et al., 2005).

Las fuentes dietéticas de compuestos fenólicos incluyen bayas, uvas, vino, té, chocolate, café y una variedad de frutas y verduras. La ingesta de polifenoles puede alcanzar cantidades de gramos, especialmente para individuos que consuman porciones múltiples de frutas, verduras, té o café todos los días. Los efectos beneficiosos de los compuestos fenólicos en la salud se deben a su capacidad para contrarrestar el estrés oxidativo y modular los procesos inflamatorios, a través de varios mecanismos de acción. El estrés oxidativo puede ser contrarrestado por los compuestos fenólicos a través de la captura de radicales libres, quelación de metales, inhibición de enzimas pro-oxidantes, activación del factor de transcripción nuclear Nrf2 por formación de radicales libres, incrementando de esta manera enzimas antioxidantes como superóxido dismutasa, catalasa, glutatión peroxidasa y glutatión

reductasa. Por otro lado, la modulación de la inflamación por parte de estos compuestos se debe a que inhibe la activación de factores de transcripción como NF-κB y MAPK, interacción con miRNAs, metilación del ADN, metilación y acetilación de histonas, bloqueo del metabolismo del acido araquidónico por inhibición de la fosforilación de PLA2, entre otros (Costa et al., 2012; Forester & Lambert, 2011; Rahman, 2008; Rahman et al., 2006; Santangelo et al., 2007).

La fibra y los compuestos fenólicos son los componentes de las frutas que más se han relacionado con el efecto inmunomodulador que puede tener el consumo frecuente de estos alimentos en la dieta, sin embargo, no se puede descartar el efecto anti-inflamatorio que puedan aportar otros fitoquímicos.

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CAPÍTULO 1: Especies vegetales colombianas con potencial anti-inflamatorio y su efecto en un modelo *in vivo* de inflamación tópica.

En este capítulo se presenta evidencia científica de la actividad anti-inflamatoria de especies vegetales utilizadas en la medicina popular colombiana. Los resultados se encuentran consignados en los productos de generación de nuevo conocimiento del 1 al 4 de esta tesis doctoral, los cuales se describen a lo largo de este capítulo.

En resumen, la evaluación de la actividad anti-inflamatoria *in vitro* de 41 especies vegetales permitió evidenciar el potencial de las especies *Physalis angulata, Mammea americana* y *Cordia alba*, las cuales fueron seleccionadas para profundizar en el estudio de sus efectos anti-inflamatorios y antioxidantes, identificando dianas moleculares asociadas a la actividad, así como determinar su capacidad de inhibir la inflamación en un modelo *in vivo*, para lo cual se empleó el modelo de edema auricular inducido por TPA en ratones.

A continuación, se presentan los productos de generación de nuevo conocimiento relacionados con esta evaluación:

Producto I. Castro, J. P., Franco, L. A., & Diaz, F. (2021). Anti-inflammatory screening of plant species from the Colombian Caribbean Coast. Journal of Applied Pharmaceutical Science, 11(04), 106-117.

Producto II: Castro, J., Salas, R., & Franco, L. Anti-inflammatory and Reactive Oxygen Species Suppressive Effects of an Extract of *Mammea americana*. Artículo en Preparación.

Producto III: Rivera, D. E., Ocampo, Y. C., Castro, J. P., Barrios, L., Diaz, F., & Franco, L. A. (2019). A screening of plants used in Colombian traditional medicine revealed the anti-inflammatory potential of *Physalis angulata* calyces. Saudi journal of biological sciences, 26(7), 1758-1766.

Producto IV: Castro, J., Rivera, D., & Franco, L. A. (2019). Topical anti-inflammatory activity in TPA-induced mouse ear edema model and *in vitro* antibacterial properties of *Cordia alba* flowers. Journal of Pharmaceutical Investigation, 49(3), 331-336.

PRODUCTO I.

ANTI-INFLAMMATORY SCREENING OF PLANT SPECIES FROM THE COLOMBIAN CARIBBEAN COAST.

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ABSTRACT

Chronic inflammation has been recognized as an underlying pathophysiological mechanism in the initiation and progression of noncommunicable diseases, representing a significant morbidity and mortality cause worldwide. The prolonged use of anti-inflammatory drugs has been associated with different adverse effects, so there is a permanent need to develop new drugs to treat these pathologies. The anti-inflammatory potential of 37 extracts coming from 31 plant species from the Colombian Caribbean coast, was evaluated determining their ability to inhibit nitric oxide (NO) production using lipopolysaccharide (LPS) activated macrophages. The most active extracts were evaluated for their effect on the production of tumor necrosis factor (TNF-α) and interleukins 1β and 6 (IL-1β and IL-6) in macrophages. Fifteen extracts showed potent inhibitory activity of the production of NO, being the extracts of *Ambrosia cumanensis*, *Trichilia hirta*, *Hyptis capitata* (leaves and seeds), *Mammea americana*, and *Crateva tapia*, the most active extracts. The extracts of *A. cumanensis* and *M. americana* were considered promising, which significantly decreased the production of all proinflammatory cytokines evaluated. The species of *A. cumanensis* and *M. americana* are a promising source of molecules with anti-inflammatory activity. They should be evaluated in *in vivo* models of inflammation, as well as perform their fractionation to identify the compounds responsible for the activity.

Key words: Natural products, noncommunicable diseases, inflammatory mediators, macrophages.

INTRODUCTION

Inflammation is a fundamental component of the host defense mechanism against noxious stimuli. This process induces the proliferation and interaction of several types of cells and the release of various chemical mediators, including prostaglandins, leukotrienes, cytokines, chemokines, and nitric oxide (NO). Although the inflammatory response is necessary for the host's defense, when it occurs in an exacerbated manner, it constitutes a triggering factor for various chronic noncommunicable diseases (Calder, 2015).

Nonsteroidal anti-inflammatories and corticosteroids are the most commonly used groups of drugs for the treatment of inflammatory processes. Still, they also have numerous adverse effects, including heartburns, gastric and duodenal ulcers, cardiovascular diseases, kidney failure, osteoporosis, increase risk of susceptibility to infections, hyperglycemia, and obesity. This represents a problem for patients' health, causing alterations that sometimes are more serious than the initial condition, in addition to increasing the costs of long-term treatments. All these reasons justify the enormous efforts made worldwide to discover new therapeutic alternatives for the treatment of inflammatory processes (Manson et al., 2009; Shi et al., 2003), being natural products one of the main objectives because they have been, and still are a fundamental source in the discovery of drugs (Killeen et al., 2014). Consequently, many investigations have been mainly directed toward the screening of plant species as a strategy in search for metabolites with anti-inflammatory activity. This search for anti-inflammatories coming from natural sources is gaining more importance today due to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) pandemic since the end of 2019, a virus that induces an exacerbated inflammatory process if adequate anti-inflammatory treatments are not received at the beginning of the infection. Colombia is one of the most mega-diverse countries in the world since it has a unique combination of geographic and topographical attributes that have allowed it to have an extraordinary diversity of flora (Gómez-Estrada et al., 2011). However, many of the medicinal properties of a high percentage of the plant species that make up the Colombian flora are still unknown.

Macrophages are one of the most important effector cells in the inflammatory response; however, during chronic inflammatory processes, they play an essential role in tissue damage in a large number of diseases that occur with inflammatory processes such as arthritis, atherosclerosis, obesity, cancer, diabetes, and inflammatory bowel disease (Kühl et al., 2015; Na et al., 2018; Treuter et al., 2017). Active macrophages produce various molecules, including the so-called reactive oxygen species and reactive nitrogen species (RNS), among which is NO, produced by the different isoforms of the enzyme nitric oxide synthase. The inducible isoform of nitric oxide synthase (iNOS) is responsible for the production of NO during the inflammatory response. The NO reacts with the superoxide radical anion ($\bullet O2^-$) to produce peroxynitrite ion ($ONOO^-$), which plays an essential role in the host's defense. Peroxynitrite is a potent oxidizing agent that exerts its effects through the mutagenesis of

microbial DNA, the inactivation of virulence factors, and the metabolic blockade in invading microorganisms, but due to its lack of specificity at high concentrations or when the synthesis occurs continuously, it produces tissue damage; this process being particularly well described in cardiovascular diseases especially in atherosclerosis (Bogdan, 2015; Lugrin et al., 2014). *In vitro* quantification of NO is inexpensive and quick in comparison with other mediators. Hence, its use to identified plant extracts with anti-inflammatory potential constitutes an important strategy that must be investigated. With the aim of exploring the ethnobotanical richness of our Caribbean region in search of new therapeutic agents with anti-inflammatory potential, in this work, we evaluated 31 plant species used in the traditional medicine of the Colombian Caribbean coast to treat various diseases related to inflammatory processes, determining their capacity to inhibit the production of the inflammatory mediator NO, in macrophages. In addition, the anti-inflammatory activity of the most active species (nitrite inhibition > 95%) was confirmed, determining their effects on the production of the proinflammatory cytokines IL-1β, IL-6, and TNF-α in LPS-activated RAW 264.7 cells.

MATERIAL AND METHODS

Reagents

Macrophages RAW 264.7 were acquired from the American Type Culture Collection (Manassas, VA). Penicillin-streptomycin, trypan blue, and lipopolysaccharide from Escherichia coli (LPS), N′-[[3-(aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride (1400W), sodium nitrite, N-[1,1-naphthyl] ethylenediamine dihydrochloride, 4-aminobenzenesulfonamide, sodium nitroprusside (SNP), dexamethasone, rofecoxib, Folin– Ciocalteu, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma Aldrich (St Louis, MO). Bromide of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) was obtained from Calbiochem® (San Diego, CA). Ethanol, dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Pittsburgh, PA). ELISA kits from eBiosciences.

Collection and identification of plant material

Plant species were collected in the Caribbean coast of Colombia. The taxonomic identification was carried out in the Guillermo Piñeres Botanical Garden of Cartagena and the Colombian National Herbarium of the National Science Institute of the National University of Colombia; voucher specimens of each species were kept in the corresponding institution.

Preparation of extracts and preliminary phytochemical analysis

The previously dried and milled plant material was subjected to extraction by maceration with 96% ethanol at room temperature (25°C ± 3°C). The obtained extract was filtered and concentrated in a rotary evaporator under reduced pressure and controlled temperature (40°C–45°C) and stored at –20°C. The following secondary metabolites' presence was determined qualitatively: alkaloids, coumarins, tannins, cardiotonic glycosides, flavonoids, saponins, triterpenes/steroids, and quinones, using a previously reported methodology (Herrera et al., 2014). Each analysis was carried out in triplicate.

Effects on the production of NO and cytotoxicity in macrophages RAW 264.7

RAW 264.7 macrophages were maintained in DMEM, enriched with 10% inactivated FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL), at 37°C and 5% CO_2. The viability of macrophages RAW 264.7, treated with different concentrations of the extracts, was determined using the method proposed by Ferrari et al. (1990), with some modifications (Ferrari et al., 1990).

The RAW 264.7 macrophages were seeded in 96-well plates (2×10^4 cells/well) and incubated for 48 hours. Subsequently, the cells were washed with phosphate buffered saline (PBS) and treated with the extracts under study at different concentrations; Triton X-100 (20%) was used as a positive cytotoxicity control. Thirty minutes later, the cells were stimulated with LPS (1 μg/ mL) and incubated for 24 hours. After this time, a 70 μL aliquot of each well was preserved for subsequent determination of nitrite levels, and the cells were rewashed with PBS, and 100 μL of the MTT solution (250 μg/mL) was added and incubated again for 4 hours. Finally, the supernatant was removed and the formazan crystals were dissolved with 100 μL of DMSO and the absorbance was determined in a microplate reader (Multiskan GO, Thermo Scientific) at DO_{550}. The evaluated extracts were considered cytotoxic when the percentage of cell survival was less than 80% (Ospina et al., 2013). The extracts' ability to inhibit NO production was determined by quantifying nitrite levels in supernatants from cell culture using the Griess reaction (Green et al., 1982). 70 μL of Griess reagent was added to 70 μL of the supernatant from the previously stored cell culture medium. The samples' absorbance was determined in a Multiskan GO microplate reader at DO_{550}, and the concentration was calculated using a standard $NaNO_2$ curve (1–200 μM). The positive control was 1400 W (10 μM), a selective inhibitor of iNOS.

NO scavenging activity

The scavenging activity of the most active extracts on NO was determined using the method described by Sreejayan and Rao (1997), with some modifications (Sreejayan and Rao, 1997). NO was generated spontaneously by SNP in an aqueous solution with physiological pH,

which in turn produces nitrite when interacting with oxygen, which can be easily determined using Griess reagent. The radical scavengers compete for the NO• radicals with the oxygen, with the consequent reduction in nitrite production. In summary, 1 mL of 10 mM SNP solution in PBS was mixed with 10 μL of the extracts under study at the maximum concentration at which they were evaluated against RAW 264.7 macrophages in the model to determine their ability to inhibit NO mediator production. The mixture was left in incubation for 4 hours at room temperature, after which 100 μL of this mixture was transferred to a 96-well plate, and 100 μL of the Griess reagent was added. The OD_{550} was determined in a microplate reader (Multiskan GO, Thermo) and compared with a standard curve of sodium nitrite to calculate nitrite concentration.

Quantification of phenolic compounds

Folin–Ciocalteu's method was used for the quantification of phenolic compounds (Del-Toro-Sánchez et al., 2014). Briefly, 30 μL of different concentrations of the extracts under study was taken and added to 150 μL of a Folin–Ciocalteu solution (0.1 M); the mixture was incubated at room temperature for 10 minutes, after which 120 μL of a sodium carbonate solution (7.5%) was added and incubated again for 2 hours. The DO_{760} was determined in a microplate reader (Multiskan GO, Thermo). The results are presented as mg of gallic acid per gram of dry extracts.

Determination of the antioxidant potential

DPPH radical scavenging activity

The DPPH• free radical scavenging capacity was determined using a standard method (Brand-Williams et al., 1995) with some modifications (Castro et al., 2019). In a 96-well microplate, 75 μL of different concentrations of the extracts were mixed with 150 μL of the DPPH methanolic solution (100 μg/mL). The mixture was incubated at room temperature for 30 minutes, after which the disappearance of the DPPH• radical at DO_{550} was determined spectrophotometrically. The results were expressed as micromoles of Trolox per gram of dry extract.

ABTS radical scavenging activity

The ABTS•+ radical scavenging activity was determined using a standard method (Re et al., 1999) with some modifications (Castro et al., 2019). The ABTS•+ radical originates by reacting ABTS (7 mM) with potassium persulfate (2.45 mM) incubated at 4°C in the dark for 16 hours. The solution of the ABTS•+ radical was diluted with ethanol to obtain an absorbance value of 0.70 ± 0.01 at 734 nm; 180 μL of this solution was mixed with 20 μL of the solutions with different concentrations of the extracts under study. The mixture was

incubated at room temperature for 30 minutes, after which the disappearance of the ABTS•+ radical to DO_{734} was determined spectrophotometrically. The results are presented as micromoles of Trolox per gram of dry extract.

Quantification of cytokines and PGE2

Macrophages RAW 264.7 were seeded in 24-well plates (2×10^5 cells/well) and incubated for 24 hours. After that time, cells were treated with the extracts and incubated for 30 minutes, activated with LPS (1 μg/ml), and incubated again for 24 hours to produce the inflammatory mediators (IL-1β, IL-6, TNF-α, and PGE2). The culture supernatants were collected and kept at −20°C until further analysis. The production of IL-1β, IL-6, TNF-α, and PGE2 was measured using a standard sandwich ELISA procedure following the manufacturer's instructions of kits from eBiosciences (San Diego, CA). Dexamethasone and rofecoxib were used as positive controls and evaluated under the same conditions.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Values of $p < 0.05$ were considered significant

RESULT AND DISCUSSION

Colombia has an enormous biological diversity and is considered one of the 17 "mega-diverse countries" in the world, according to the United Nations Environment Program. Large amounts of data about Colombia's ecosystems are being collected in the "Colombia BIO" expeditions, including novel biodiversity in previously unexplored regions due to the internal conflict. Unfortunately, for a high percentage of these species, there is sparse knowledge, which has avoided taking advantage of the benefits in the food, medicine, and industry (De Vega et al., 2020). An alternative way to take advantage of these biological resources is the practice of bioprospecting, which implies the interaction between different types of knowledge, especially ancestral knowledge that local and indigenous communities in South American countries have, with the scientific knowledge contributed by the academic and industrial sectors, especially the pharmaceutical industry (Duarte and Velho, 2008). To contribute to the bioprospecting of our natural resources and in accordance with the permanent need to develop new drugs with anti-inflammatory properties, in this work, we selected 31 plant species used in the popular medicine of the Colombian Caribbean coast to assess its anti-inflammatory potential.

Collection, identification of plant material, and extract preparation

Information on the collected species, including common names, the part of the plant evaluated, the collection site, the species' registration codes, and the yields of the extracts obtained, is presented in Table 1.1 and their preliminary phytochemical analysis is presented in Table 1.2

Effect on RAW 264.7 cell viability

As shown in Figure 1.1, only the extracts of *Momordica charantia*, *Inga vera*, *Ambrosia cumanensis* (seeds and leaves), *Trichilia hirta*, *Tabernaemontana cymosa* (seeds), and *Anacardium occidentale* showed toxicity (cell viability under 80%) on RAW 264.7 macrophages at 100 μg/mL. Therefore, these extracts were evaluated to determine their highest nontoxic concentration and their anti-inflammatory effect on macrophages RAW 264.7 was securely evaluated. The selected concentration of each extract can be seen in Table 1.3.

Effect on NO production in RAW 264.7 macrophages

The anti-inflammatory potential of the extracts was established by determining their effect on the release of the NO inflammatory mediator in the RAW 264.7 macrophage cell line. When macrophages are stimulated, they produce NO, which is important in protecting the organism against viruses and pathogenic microorganisms (Bogdan, 2015; Wallace, 2005). The antiviral or antimicrobial effect of NO and other RNS such as peroxynitrite is related to their ability to react with structural elements, components of the replication machinery, metabolic enzymes, and molecules associated with the virulence of infectious pathogens (Bogdan, 2015). However, NO when it is in high concentrations or when it is produced continuously, as it happens during chronic inflammatory processes, can produce harmful results for the tissues, being able to react with the superoxide free radical (O2-) to generate the peroxynitrite anion ($ONOO^-$), from which the formation of hydroxyl radicals (•OH), carbonate ($CO_3•^-$), and nitrogen dioxide ($•NO_2$) can be derived. These free radicals can interact with proteins, lipids, and nucleic acids, promoting various molecular modifications responsible for altering the cells' biological functions, leading to the appearance of various chronic diseases. Therefore, the iNOS inhibitors could have a therapeutic application in conditions that occur with chronic inflammatory processes such as arthritis, inflammatory bowel disease, and cancer (Bogdan, 2015; Lugrin et al., 2014).

Table 1.1. Information on vegetable species in the study.

Scientific name	Family	Local name	Part used	Voucher number	Extract yields (%)	Collection site
Crotalaria retusa L.	Fabaceae	Cascabelito	Seed	JBC 12007	8.7	Galerazamba, Bolívar.
Heliotropium indicum L.	Boraginaceae	Rabo de alacran	Seed	JBC 3691	6.8	(10°47'22"N, 75°15'35"W)
Mammea americana L.	Calophyllaceae	Mamey	Leaves	JBC 467	7.3	
Murraya exotica L.	Rutaceae	Azahar de la india	Leaves	COL 538418	6.3	
Pedilanthus tithymaloides (L.) Poit.	Euforbiaceae	Pitamorreal	Leaves	JBC 1018	8.1	
Momordica charantia L.	Cucurbitaceae	Balsamina	Leaves	JBC 793	9.8	San Basilio de Palenque, Bolívar. (10°6'12"N, 75°11'56"W)
Ambrosia cumanensis Kunth.	Asteraceae	Artemisa	Leaves	COL 538448	5.8	San Bernardo del viento,
			Seed	COL 538448	9.8	Córdoba.
Anacardium occidentale L.	Anacardiaceae	Marañon	Seed	JBC 4431	7.5	(9°21'18"N, 75°57'16"W)
Annona squamosa L.	Annonaceae	Guanabana	Seed	JBC 4431	8.0	
Bursera graveolens Kunth.	Burseraceae	Caraña	Bark	JBC 5115	7.9	
Bursera simaruba (L.) Sarg	Burseraceae	Almácigo	Bark	JBC 4458	6.5	
Caesalpinia coriaria (Jacq.) Willd.	Fabaceae	Dividivi	Fruit	COL 538422	8.2	
Capparis odoratissima (Jacq.) Hutch.	Capparaceae	Olivo	Leaves	JBC 1492	7.6	
Chenopodium ambrosioides L.	Amaranthaceae	Paico	Leaves	JBC 4005	9.4	
Cecropia peltata L.	Urticaceae	Yarumo	Leaves	JBC 1383	7.8	
			Bark	JBC 1383	7.8	
Crateva tapia L.	Capparaceae	Naranjuelo	Leaves	JBC 12017	6.4	
Diospyros inconstans Jacq.	Ebenaceae	Caimitillo	Bark	JBC 1438	10.2	
Eryngium foetidum L.	Apiaceae	Culantro	Leaves	COL 538419	8.8	
Gustavia superba (Kunth) O.Berg.	Lecythidaceae	Membrillo	Leaves	JBC 1382	6.8	
Hippomane mancinella L.	Euphorbiaceae	Manzanillo	Leaves	JBC 2478	6.7	
			Fruit	JBC 2478	5.4	
			Seed	JBC 2478	8.7	
Hura crepitans L.	Euphorbiaceae	Ceiba blanca	Bark	JBC 788	7.2	
Hyptis capitata Jacq.	Lamiaceae	Botón negro	Leaves	JBC 2478	10.4	
			Seed	JBC 2478	9.8	
Inga vera Willd.	Fabaceae	Guama	Seed	JBC 17149	8.1	
Piper peltatum L.	Piperaceae	Santa María	Leaves	JBC 1438	4.2	
Ruellia tuberosa L.	Acanthaceae	Campana	Leaves	JBC 3932	6.8	
Sarcostemma clausum (Jacq.) Schult.	Apocynaceae	Bejuco de sapo	Leaves	JBC 2502	7.3	
Sterculia apetala (Jacq.) H. Karst.	Malvaceae	Camajuro	Seed	COL 538417	7.8	
Tabebuia ochracea (Cham.) Standl.	Bignoniaceae	Polvillo	Bark	JBC 5153	6.8	
Tabernaemontana cymosa Jacq.	Apocynaceae	Bola de puerco	Seed	JBC 3243	9.6	
			Bark	JBC 3243	7.3	
Thevetia peruviana (Pers.) Merr.	Apocynaceae	Cavalonga	Flowers	JBC 66	7.4	
Trichilia hirta L.	Meliaceae	Jobo macho	Seed	JBC 4330	7.9	

The taxonomic identification was carried out in the Guillermo Piñeres Botanical Garden of Cartagena (Voucher JBC) and the Colombian National Herbarium of the National Science Institute of the National University of Colombia (Voucher COL). The yields were calculated as follows: [Dry Concentrated Extract (g)/Dry Material(g)] × 100.

The effect of the extracts under study on the inhibition of NO production in macrophages RAW 264.7 is presented in Table 1.3. The extracts were classified according to their activity as active (% inhibition ≥ 60), moderate (60 > % inhibition > 40), mild (40 > % inhibition > 20), and inactive (% inhibition < 20). Fifteen extracts corresponding to 40.5% of all the extracts evaluated were classified as actives, being the extracts of *A. cumanensis* (seeds), *T. hirta* (seeds), *Hyptis capitata* (seeds and leaves), *Mammea americana* (leaves), and *Crateva tapia* (leaves) the most active, with inhibition values greater than 95%, presenting itself as powerful inhibitors of the production of NO *in vitro*, and therefore, they could be the basis for developing new therapies to prevent the oxidation of macromolecules mediated by the NO during the chronic inflammatory processes; characteristic condition of pathologies such as cancer, diabetes, and inflammatory bowel disease. Plant species to which the most active extracts belong are widely used in traditional medicine to treat various health conditions

(Table 1.4), including some related to chronic inflammatory processes. It is important to highlight the potent activity shown by the extracts of *A. cumanensis* (seeds) and *T. hirta* (seeds), which exerted their inhibitory effect at lower concentrations (25 and 12 μg/ml) than the required for the other extracts (100 μg/ml). The rest of evaluated extracts showed moderate activity (4 extracts, 10.8%) or mild activity (8 extracts, 21.6%) or were inactive (10 extracts, 27.0%).

Table 1.2. Phytochemical characterization of vegetable species in the study.

Scientific name	Part used	Metabolite							
		Alkaloids	Coumarins	Tannins	Glycosides	Flavonoids	Saponins	Terpenes/ Steroids	Quinones
Ambrosia cumanensis	Leaves	-	-	-	+	-	+	+	+
	Seed	+	+	+	-	+	-	+	-
Anacardium occidentale	Seed	-	+	+	-	+	+	+	-
Annona squamosa	Seed	+	+	+	-	+	+	+	-
Bursera graveolens	Bark	-	+	-	+	+	-	-	+
Bursera simaruba	Bark	+	-	-	-	+	+	+	+
Caesalpinia coriaria	Fruit	-	-	+	+	-	-	-	+
Capparis odoratissima	Leaves	+	+	-	-	-	-	+	-
Chenopodium ambrosioides	Leaves	-	-	-	-	+	-	-	-
Cecropia peltata	Leaves	+	-	+	+	+	+	+	+
	Bark	+	+	-	+	+	+	+	-
Crataeva tapia	Leaves	+	+	-	+	-	+	-	-
Crotalaria retusa	Seed	+	-	-	-	+	+	-	-
Diospyros inconstans	Bark	+	+	+	-	-	+	+	-
Eryngium foetidum	Leaves	-	-	-	+	-	-	+	+
Gustavia superba	Leaves	+	-	-	-	-	-	+	-
Heliotropium indicum	Seed	-	-	-	+	+	-	-	+
Hippomane mancinella	Leaves	+	+	+	+	+	-	+	-
	Fruit	-	+	-	-	-	+	+	-
	Seed	+	+	+	+	+	-	-	+
Hura crepitans	Bark	+	+	+	+	+	-	-	+
Hyptis capitata	Leaves	+	+	+	-	+	+	-	-
	Seed	+	+	-	+	+	-	-	+
Inga vera	Seed	-	+	-	-	+	-	-	-
Mammea americana	Leaves	-	+	+	+	+	+	+	-
Momordica charantia	Leaves	+	-	-	+	-	-	+	-
Murraya exotica	Leaves	+	+	-	-	+	+	+	-
Pedilanthus tithymaloides	Leaves	+	-	+	+	+	-	-	+
Piper peltatum	Leaves	+	-	+	+	+	+	+	+
Ruellia tuberosa	Leaves	-	-	+	-	+	+	+	+
Sarcostemma clausum	Leaves	-	-	-	+	+	-	-	+
Sterculia apetala	Seed	+	+	+	-	+	-	-	-
Tabebuia ochracea	Bark	-	-	+	-	+	-	-	+
Tabernaemontana cymosa	Seed	+	-	-	+	+	+	+	+
	Bark	+	+	+	+	-	+	-	-
Thevetia peruviana	Flowers	-	-	-	+	+	-	-	+
Trichilia hirta	Seed	+	+	-	+	-	+	+	-

+: presence and −: not detected.

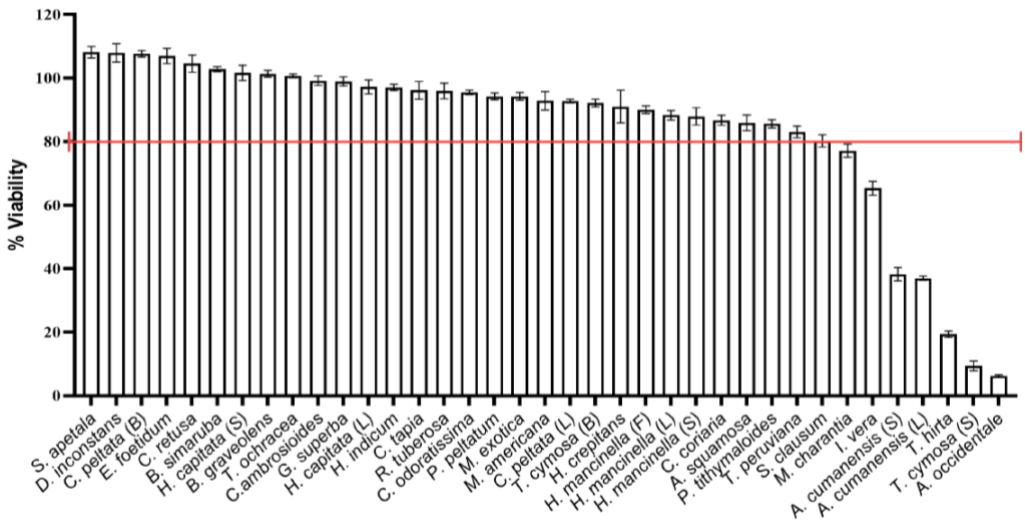

Figure 1.1. Effect of extracts on cell viability of RAW 264.7 macrophages. (S): seeds; (L): leaves; (B) bark; (F) fruit. Cytotoxicity was measured with MTT assay. Each value represents mean ± SEM (n = 9)

Quantification of phenolic compounds and determination of free radical scavenging capacity

Phenolic compounds are widely known for their antioxidant effects, mainly associated with their free radical scavenging capacity and their impact on the decrease of various inflammatory mediators, including NO (Conforti and Menichini, 2011; Fernandez-Panchon et al., 2008). The content of total phenolic compounds, as well as their scavenger activity on DPPH and ABTS free radicals, was quantified in the extracts considered as active, observing a strong correlation between the presence of the phenolic compounds and the scavenger effect of free radicals DPPH (r = 0.86) and ABTS (r = 0.91); in fact, extracts of *A. occidentale* (seeds), *Bursera simaruba* (bark), *H. capitata* (seeds), and *M. americana* (leaves) showed the highest content of phenolic components as well as the best DPPH• and ABTS•+ radical scavenging activity (Table 1.5). With respect to the NO scavenging activity, a low correlation (r = 0.27) between the phenolic compounds content and this scavenging activity was observed (Table 1.6). Therefore, the effect on the inhibition of NO• production in LPS-induced RAW 264.7 macrophages is related to the impact on cells and not with the scavenging effects of NO by the active extracts.

Table 1.3. Effect of evaluated extracts on the production intracellular of NO in LPS-stimulated RAW 264.7 macrophages.

Scientific name	Part used	Concentration (µg/mL)	Cell viability (%)	Nitrite inhibition (%)	Classification
Ambrosia cumanensis	Seed	25	95.8 ± 3.1	113.6 ± 0.8	Active
Trichilia hirta	Seed	12	91.0 ± 3.3	108.2 ± 1.4	
Hyptis capitata	Leaves	100	97.2 ± 2.2	101.6 ± 0.7	
Hyptis capitata	Seed	100	101.6 ± 2.4	100.3 ± 0.7	
Mammea americana	Leaves	100	92.8 ± 2.9	97.8 ± 2.4	
Crataeva tapia	Leaves	100	96.1 ± 2.8	95.0 ± 5.8	
Ambrosia cumanensis	Leaves	25	94.0 ± 0.7	93.0 ± 0.8	
Bursera simaruba	Bark	100	102.8 ± 0.8	92.8 ± 1.1	
Murraya exotica	Leaves	100	94.2 ± 1.2	91.6 ± 5.3	
Eryngium foetidum	Leaves	100	106.9 ± 2.4	90.4 ± 1.8	
Annona squamosa	Seed	100	85.9 ± 2.5	74.2 ± 3.7	
Heliotropium indicum	Seed	100	97.0 ± 1.0	66.6 ± 3.8	
Cecropia peltata	Leaves	100	92.7 ± 0.6	66.2 ± 1.9	
Anacardium occidentale	Seed	50	106.3 ± 5.7	60.3 ± 1.8	
Chenopodium ambrosioides	Leaves	100	99.1 ± 1.5	60.0 ± 2.0	
Hippomane mancinella	Leaves	100	88.3 ± 1.5	48.2 ± 2.8	Moderately active
Cecropia peltata	Bark	100	107.6 ± 1.0	44.8 ± 2.7	
Tabernaemontana cymosa	Bark	100	92.1 ± 1.2	41.0 ± 4.8	
Diospyros inconstans	Bark	100	107.9 ± 2.9	40.7 ± 2.8	
Ruellia tuberosa	Leaves	100	95.9 ± 2.5	38.1 ± 1.9	Mildly active
Capparis odoratissima	Leaves	100	95.4 ± 0.7	34.2 ± 3.6	
Momordica charantia	Leaves	50	97.4 ± 3.2	30.3 ± 2.3	
Hura crepitans	Bark	100	91.0 ± 5.1	27.1 ± 2.3	
Piper peltatum	Leaves	100	94.2 ± 1.1	22.6 ± 3.6	
Caesalpinia coriaria	Fruit	100	86.7 ± 1.6	21.2 ± 3.4	
Gustavia superba	Leaves	100	98.9 ± 1.4	20.8 ± 1.9	
Crotalaria retusa	Seed	100	104.5 ± 2.7	20.2 ± 2.9	
Hippomane mancinella	Seed	100	87.9 ± 2.7	17.8 ± 1.9	Inactive
Sterculia apetala	Seed	100	108.1 ± 1.8	15.1 ± 3.7	
Inga vera	Seed	25	84.8 ± 2.8	13.5 ± 2.0	
Tabernaemontana cymosa	Seed	50	84.8 ± 0.7	11.3 ± 1.9	
Bursera graveolens	Bark	100	101.3 ± 1.1	8.1 ± 1.0	
Sarcostemma clausum	Leaves	100	80.2 ± 1.9	7.0 ± 3.1	
Hippomane mancinella	Fruit	100	90.0 ± 1.2	5.2 ± 1.6	
Thevetia peruviana	Flowers	100	83.0 ± 1.8	0.2 ± 5.6	
Pedilanthus tithymaloides	Leaves	100	85.6 ± 1.3	0.0	
Tabebuia ochracea	Bark	100	100.6 ± 0.6	0.0	

The results represent the mean ± SEM (n = 12) from three independent experiments. Active (% inhibition ≥ 60), moderate (60 > % inhibition > 40), mild (40 > % inhibition > 20), and inactive (% inhibition < 20).

In addition, phenolic compounds content showed a low correlation (r = 0.10) with the inhibition of the NO production by the macrophages RAW 264.7, in such a way that there is no direct relationship between the activity shown by extracts and the total phenolic content present in the extracts; for instance, extracts such as *A. cumanensis* (seeds and leaves), *T. hirta* (seeds), *H. capitata* (leaves), *C. tapia* (leaves), *Murraya exotica* (leaves), and *Eryngium foetidum* (leaves) that showed potent inhibitory activity of NO production in the cell line (% inhibition ≥ 90%) had a low content of phenolic compounds in their composition (< 30 mg of gallic acid/g of extract), while the extract of *A. occidentale* (seeds) that showed the highest content of phenolic compounds only inhibited in 60.28% of the NO in the cells. This low correlation is not sufficient reason to rule out that this group of metabolites can be associated with this biological activity since this not only depends on the concentration at which the phenolic compounds are found but also on their structure, of which there are more than 8,000 known structures, in a range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins (Conforti and Menichini, 2011; Sofi and

Nabi, 2018). In fact, several phenolic compounds, including resveratrol, isovitexin, isoliquiritigenin, baicalin, baicalein, wogonin, apigenin, and luteolin, have shown the ability to inhibit the iNOS expression and NO production in LPS-induced RAW 264.7 macrophages (Conforti and Menichini, 2011).

Table 1.4. Summary of ethnopharmacological antecedents of the most active plant species.

Scientific name	Part used	Mode of preparation	Principal medicinal indication	Way of Administration	References
Ambrosia cumanensis	Leaves (fresh)	Decoction	Common cold	Bath	(Gómez-Estrada et al., 2011)
			Intestinal parasites	Orally	
		Poultice	Headache	Applied locally	
Crataeva tapia	Bark	Paste of bark powder	Arthritis	Applied locally	(Sharma et al., 2013)
		Decoction	Abscess	Orally	
			Hypoglycemic		
	Leaves and stem	Decoction	Dysentery/Diarrhoea	Orally	
Hyptis capitata	Whole plant	Juice	Malaria	Orally	(Biswas et al., 2010)
	Leaves, flowers and roots	Decoction	Renal stones, liver disease, wounds, cough, shortness of breath, diabetes	Orally	(Gailea et al., 2016)
Mammea americana	Seed	Macerated	Lice control	Applied locally	(Germosén Robineau et al., 2017)
	Leaves	Poultice	Rheumatism		
Trichilia hirta	Leaves and branch	Decoction	Flu	Orally	(Beyra et al., 2004)

Table 1.5. Quantification of phenolic compounds and scavenging effect on DPPH and ABTS radicals of the active extracts.

Scientific name	Part used	Phenolic compounds (mg of gallic acid/ g of extract)	DPPH (µmoles trolox / g of extract)	ABTS (µmoles trolox / g of extract)
Anacardium occidentale	Seed	142.9 ± 2.4	1652.2 ± 28.5	1028.1 ± 19.4
Bursera simaruba	Bark	106.3 ± 2.4	831.6 ± 14.6	1551.3 ± 22.2
Mammea americana	Leaves	97.3 ± 2.6	323.5 ± 6.7	1155.0 ± 14.9
Hyptis capitata	Seed	96.8 ± 0.9	279.2 ± 3.1	769.1 ± 10.3
Cecropia peltata	Leaves	53.0 ± 1.3	176.6 ± 4.1	207.3 ± 4.4
Hyptis capitata	Leaves	30.0 ± 0.7	161.8 ± 1.4	158.8 ± 2.6
Ambrosia cumanensis	Leaves	26.4 ± 0.5	75.6 ± 1.8	51.8 ± 2.1
Ambrosia cumanensis	Seed	23.3 ± 0.5	43.9 ± 0.7	127.9 ± 3.4
Chenopodium ambrosioides	Leaves	19.8 ± 0.3	43.5 ± 0.4	77.2 ± 0.9
Eryngium foetidum	Leaves	19.7 ± 6.2	96.3 ± 1.0	220.6 ± 3.3
Trichilia hirta	Seed	15.7 ± 0.9	11.5 ± 0.2	23.0 ± 0.4
Murraya exótica	Leaves	14.8 ± 0.6	14.0 ± 0.2	64.7 ± 0.8
Crataeva tapia	Leaves	7.5 ± 0.1	15.7 ± 0.2	45.5 ± 0.5
Heliotropium indicum	Seed	3.3 ± 0.1	nd	34.8 ± 0.2
Annona squamosa	Seed	1.2 ± 0.0	nd	nd

The results represent the mean ± SEM (n = 9) from three independent experiments. nd = not determined.

Determination of inflammatory mediators

The most active extracts (nitrite inhibition > 95%) with anti-inflammatory potential were evaluated to identify their effects on the inflammatory mediators IL-6, TNF-α, and IL-1β, which is the same as NO, increase their production by activating the transcription factor NF-κB in LPS-induced macrophages (Girón et al., 2010). Results show that all evaluated extracts significantly decreased the production of IL-6, being the extracts of *A. cumanensis* (seeds) and *T. hirta* (seeds) the most potent, with inhibition percentages greater than 90%, with

activity higher than shown by dexamethasone (68%) used as a reference drug (Figure 1.2.A). Regarding the effect on TNF-α, except for the extract of *H. capitata* (leaves), all others significantly inhibited the production of this mediator with similar percentages to that shown by the reference drug (Figure. 1.2.B). Finally, the results presented in Figure 1.2.C show that only *A. cumanensis* (seeds) and *M. americana* (leaves) extracts inhibited the production of IL-1β significantly. Inflammatory mediators IL-6, IL-1β, and TNF-α are produced by many cell types, mainly macrophages and mast cells. They have several roles in the inflammatory response, including activation of the endothelium and leukocytes and induction of the acute phase response (Medzhitov, 2008).

Table 1.6. Scavenging effect of the active extracts on nitric oxide.

Scientific name	Part used	Phenolic compounds (mg of gallic acid/ g of extract)	Concentration (µg/mL)	% Scavenging NO
Anacardium occidentale	Seed	142.9 ± 2.4	50	0.0
Bursera simaruba	Bark	106.3 ± 2.4	100	12.0 ± 2.0
Mammea americana	Leaves	97.3 ± 2.6	100	24.9 ± 1.0
Hyptis capitata	Seed	96.8 ± 0.9	100	14.0 ± 1.1
Cecropia peltata	Leaves	53.0 ± 1.3	100	22.1 ± 1.2
Hyptis capitata	Leaves	30.0 ± 0.7	100	10.0 ± 0.5
Ambrosia cumanensis	Leaves	26.4 ± 0.5	25	4.9 ± 0.7
Ambrosia cumanensis	Seed	23.3 ± 0.5	25	10.8 ± 1.8
Chenopodium ambrosioides	Leaves	19.8 ± 0.3	100	10.5 ± 0.7
Eryngium foetidum	Leaves	19.7 ± 6.2	100	15.3 ± 1.4
Trichilia hirta	Seed	15.7 ± 0.9	12	4.3 ± 0.4
Murraya exotica	Leaves	14.8 ± 0.6	100	3.0 ± 0.3
Crataeva tapia	Leaves	7.5 ± 0.1	100	10.3±0.7
Heliotropium indicum	Seed	3.3 ± 0.1	100	2.3 ± 0.2
Annona squamosa	Seed	1.2 ± 0.0	100	0.0

The results represent the mean ± SEM (n = 9) from three independent experiments.

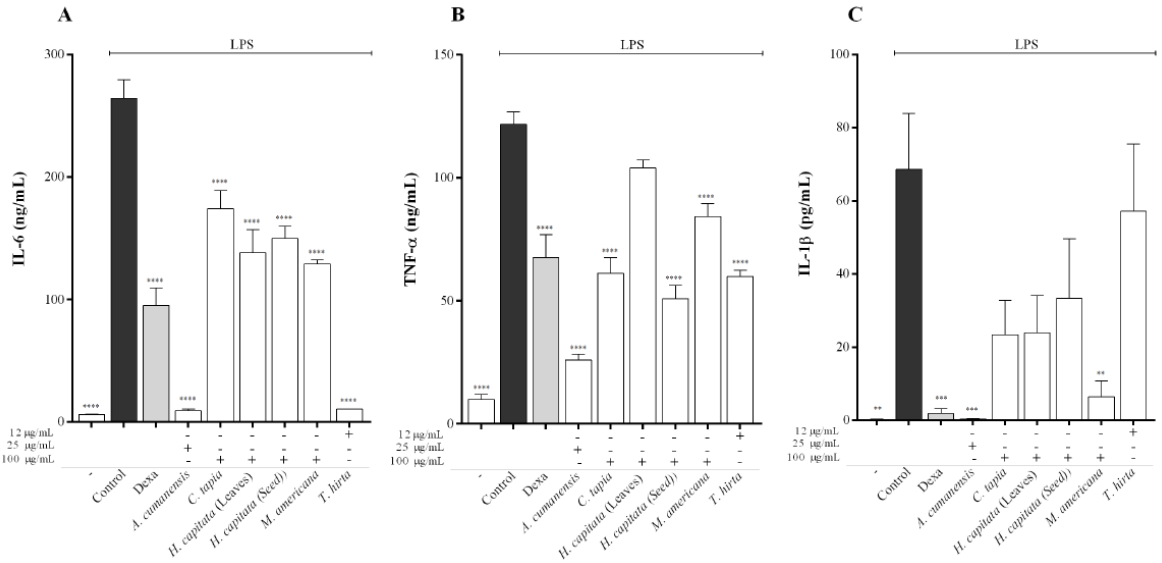

Figure 1.2. Effect of extracts on intracellular production of IL-6 (A), TNF-α (B), and IL-1β (C) in LPS-stimulated RAW 264.7 macrophages (1 µg/ml) for 24 hours. −: unstimulated cells. Dexamethasone (20 µM) was used as a positive control. Results represent the mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 ANOVA statistically significant compared with LPS-treated group).

The simultaneous inhibition of the production of these proinflammatory cytokines, as in the case of the extracts of *A. cumanensis* (seeds) and *M. americana* (leaves) (Figure. 1.2), is a key point as a pharmacological target in the treatment of inflammation. Therefore, we evaluated the concentration-dependent effect of these two extracts on these proinflammatory cytokines. Figure 1.3. shows that the *M. americana* extract showed a significant concentration-dependent effect on all mediators with particular emphasis on the NO and IL-1β, even to the lower concentration tested of 50 μg/mL. *M. americana* is a species recognized for its high content of coumarins (Crombie et al., 1987; Yang et al., 2005). Studies of this species in terms of its health benefits are limited. Some studies have been conducted on its properties related to the use of leaves, seeds, and bark as antibacterial, anthelmintic, antiviral, and antimalarial (Gómez-Calderón et al., 2017; González-Stuart, 2011; Toma et al., 2005). Until now, there are no studies that validate the anti-inflammatory activity of Mamey (*M. americana*); therefore, the present work is an important contribution to the knowledge of the anti-inflammatory properties of this species.

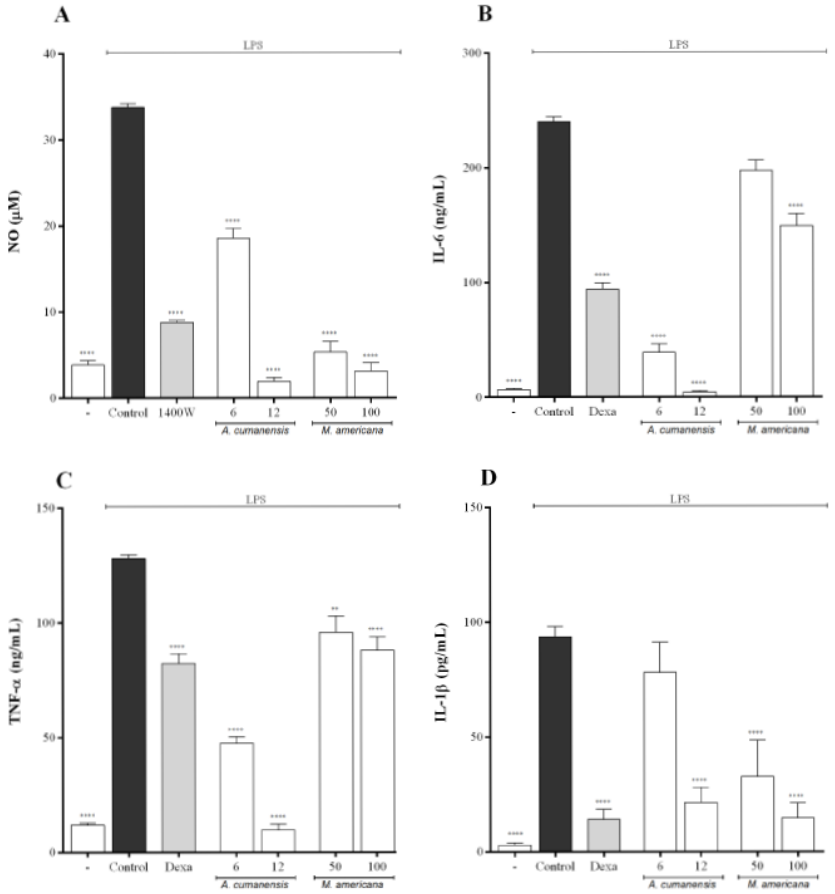

Figure 1.3. Effect of promissory extracts of *A. cumanensis* (seeds) and *M. americana* (leaves) on the production of NO (A), IL-6 (B), TNF-α (C), and IL-1β (D) in LPS-stimulated RAW 264.7 macrophages (1 μg/ml) for 24 hours. −: unstimulated cells. 1400W (10 μM) and dexamethasone (20 μM) were used as a positive control. Results represent the mean ± SEM (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ ANOVA statistically significant compared with LPS-treated group).

On the other hand, *A. cumanensis* (seeds) extract exerts its effects even at concentrations as low as 6 and 12 μg/ml in all inflammatory mediators evaluated (Figure 1.3). *Ambrosia cumanensis* is recognized for its high content of sesquiterpene lactones, compounds widely recognized for their anti-inflammatory properties (Jimenez-Usuga et al., 2016). The sesquiterpene lactones coronopilin and damsin isolated from *Ambrosia arborescens* modulate the activation of the transcription factor NF-κB, decreasing the production of IL-6, while ambrosanolide, a type of sesquiterpene lactone present in *Ambrosia psilostachya*, inhibits the production of NO in mouse peritoneal macrophages (Lastra et al., 2004; Svensson et al., 2018).

Considering the powerful activity shown by the *A. cumanensis* (seeds) extract on the evaluated cytokines, we decided to determine this extract's effect on the proinflammatory mediator PGE2 in macrophages RAW 264.7. Similar to the effect observed on cytokines, this extract inhibited LPS-induced PGE2 production in a concentration-dependent manner even at concentrations as low as 6 and 12 μg/mL, with activity at 12 μg/ mL similar to that exerted by rofecoxib used as a control (Figure 1.4.). PGE2 is an essential homeostatic factor that plays an important role in the modulation of the inflammatory and immune response through the regulation of cytokine production, leukocyte migration, proliferation, and differentiation (Díaz-Muñoz et al., 2012; Kalinski, 2012).

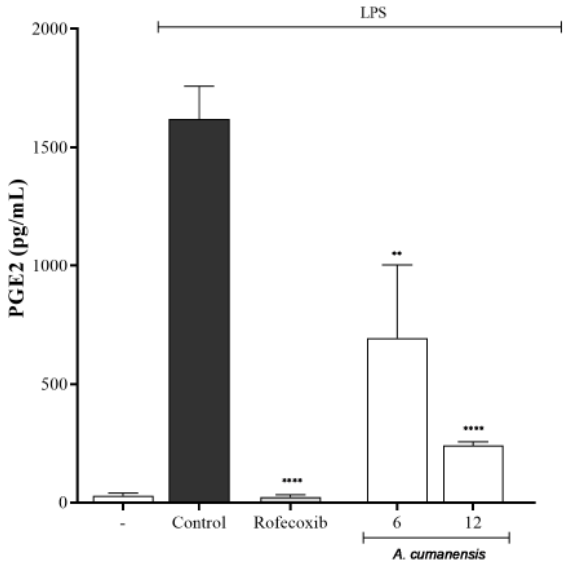

Figure 1.4. Effect of promissory extracts of *A. cumanensis* (seeds) on the production of PGE2 in LPS-stimulated RAW 264.7 macrophages (1 μg/mL) for 24 hours. −: unstimulated cells. Rofecoxib (10 μM) was used as positive control. Results represent the mean ± SEM (**p < 0.01; ****p < 0.0001 ANOVA statistically significant compared with LPS-treated group).

Conclusion

Our results show the anti-inflammatory potential of 15 extracts of 13 plant species of the Colombian Caribbean coast, which significantly inhibit the production of the NO inflammatory mediator. Extracts of the species *A. cumanensis* and *M. americana* are the most promising, inhibiting the production of all evaluated inflammatory mediators significantly, constituting a valuable biological resource of the Colombian diversity on which bioprospecting must be applied to develop new therapeutic alternatives for the treatment of diseases that occur with inflammatory processes.

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PRODUCTO II:

ANTI-INFLAMMATORY AND REACTIVE OXYGEN SPECIES SUPPRESSIVE EFFECTS OF AN EXTRACT OF *MAMMEA AMERICANA*

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ABSTRACT

The inflammatory process is one of the body's natural response mechanisms against harmful stimuli; however, when it is persistent, it gives rise to severe pathologies such as osteoarthritis, rheumatoid arthritis, gout, asthma, inflammatory bowel disease, diabetes, cancer; diseases that represent an important cause of morbidity and mortality worldwide. The species *Mammea americana* is used in the popular medicine of Colombia for the treatments of inflammation and fever. The aim of this work was to evaluate the anti-inflammatory and antioxidant potential of the total ethanolic extract obtained from the leaves of *Mammea americana*. The anti-inflammatory effect was evaluated using the *in vivo* model of TPA-induced mouse ear edema and the antioxidant potential using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method in RAW 264.7 mouse macrophages. The extract of *Mammea americana* significantly decreased the edema, the levels of inflammatory mediators (TNF-α, IL-1β, IL-6, PGE2), and the activity of the MPO enzyme in the mice ear treated with the irritant agent TPA (13-acetate-12-O-tetradecanoylphorbol). This extract also inhibited the increase of reactive oxygen species (ROS) in RAW 264.7 macrophages. The results of this project show the potential usefulness of the leaves of *Mammea americana* to counteract inflammation and oxidative stress.

Key words: *Mammea americana,* Anti-inflammatory, inflammatory mediators, Antioxidant, reactive oxygen species.

INTRODUCTION

Inflammation is understood as a variety of molecular, cellular, and vascular phenomena intended for the body to respond to various aggressions or stimuli caused by external agents that can cause damage to different organs (Rubio-Perez & Morillas-Ruiz, 2012). The inflammatory response is mediated by cells of the immune system, such as polymorphonuclear leukocytes and macrophages, whose activation induces the production

of cytokines, lipid mediators, proteolytic enzymes, and reactive oxygen species (ROS) (Chrobok et al., 2017). Sustained inflammation leads to the overproduction of reactive oxygen species (ROS), which can activate the process of damage and deterioration of target cells and organs (Khansari et al., 2009; Lavrovsky et al., 2000).

Systematic reviews have shown an association between inflammatory biomarkers, including inflammatory cytokines (TNF-α, IL-1β, IL-6), pro-inflammatory enzymes (COX-2), among other mediators; with the appearance and progression of various chronic non-communicable diseases (Prasad et al., 2012). Some of these biomarkers of inflammation can be induced by reactive oxygen species (Khansari et al., 2009; Lavrovsky et al., 2000). In this sense, inhibitors of pro-inflammatory cytokine production and/or reactive oxygen species are considered a useful strategy to reduce the incidence of chronic non-communicable diseases or prevent their progression.

Since time immemorial, plants have been used for medicinal purposes, in fact plant species were for many years the only source of medicines. This practice is still valid, despite our dependence on modern medicine and the great advances in synthetic drugs; due to the immense variety of functionally relevant secondary metabolites of plant species (Dushenkov & Raskin, 2008; Kingston, 2011). In fact, according to estimates by the World Health Organization (WHO), about 80% of the population of developing countries depends on plant preparations for the therapy of diseases (Dushenkov & Raskin, 2008).

Mammea americana is an evergreen tree, which has been classified as belonging to the Calophyllaceae family, is native to the West Indies and northern South America (Yahia & Guttierrez-Orozco, 2011). *Mammea americana* in traditional medicine is very diverse and generally includes all parts of the plant. The leaves are used as insecticides to lower blood pressure and to treat rheumatism (Lemus et al., 2021). Additionally, *in vitro* studies where the effect of an ethanol extract of *Mammea americana* leaves was studied showed that it could free radical scavenging and significantly reduce NO, TNF-α, IL-1β, and IL-6. Taking into account this background in this work, we evaluated in vivo the anti-inflammatory effect of the total ethanolic extract obtained from the leaves of *Mammea americana* and, his ability *in vitro* to decrease ROS levels.

MATERIALS AND METHODS

Chemicals

12-O-tetradecanoyl-phorbol-13-acetate (TPA), indomethacin, ethylenediaminetetraacetic acid (EDTA), O-dianisidine, quercetin, Dulbecco's modified eagle medium (DMEM), penicillin–streptomycin, trypan blue, lipopolysaccharide (LPS), N-([3 (aminomethyl)phenyl]meth yl) ethanimidamide dihydrochloride (1400W), sodium nitrite, N-

(1,1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, hematoxylin-eosin and 2′,7′-dichlorofluorescein diacetate (DCFH-DA), were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethylsulfoxide, acetone and ethanol, were obtained from Merck (Kenilworth, NJ, USA) and Carlo Erba (Val-de-Reuil, France). Hexadecyltrimethylammonium (HTAB), hydrogen peroxide (H_2O_2), bromide of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) from Calbiochem_ (San Diego, CA, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Gaithersburg, MD, USA). All other chemicals and reagents used were of analytical grade.

Plant material, preparation of the extract, and phytochemical screening

The plant material was collected in Turbana-Bolívar (10°24′0″ N, 75°30′0″ O) and plant was identified by a botanist at the Herbarium of the University of Antioquia (HUA, Medellin, Antioquia, Colombia) where voucher specimens was deposited (HUA 201954).

The leaves of *Mammea americana* were dried at room temperature, powdered, and extracted by maceration with ethanol at room temperature until exhaustion. The crude ethanolic extract was filtered and the solvent evaporated under reduced pressure at 40±5ºC using a rotary evaporator (Heidolph VV 2000, Heidolph, Kelheim, Germany). The phytochemical screening for the presence of coumarins, tannins, cardiotonic glycosides, phenolic compounds, flavonoids, Saponins, and Triterpenes/Steroids was performed as reported by Herrera et al. (2014).

***In vivo* anti-edema activity**

Experimental animals

Female ICR mice (20–25 g) provided by the Instituto Nacional de Salud (Bogotá, Colombia) were allowed to acclimatize for at least two weeks before use and fed standard rodent food and water ad libitum. They were housed in filtered-capped polycarbonate cages and kept in a controlled environment (22±3 ºC and relative humidity: 65 to 75%), under a 12 h light/darkness cycle. All experiments were designed and conducted in accordance with the European Union regulations (CEC council 86/809) and approved by the Ethics Committee of the University of Cartagena (Minutes No. 32 August 4, 2011).

TPA-induced acute ear edema

The edema was induced by topical application of TPA (2.5 µg/ear) in acetone, according to the method described by De Young et al., 1989 (De Young et al., 1989). Groups (n = 6) were treated on the inner and outer surfaces of the right ear with the acetone dissolved treatment (1 or 0.5 mg in 20 µL of the vehicle) or indomethacin (0.5 mg in 20 µL of the vehicle), as a

drug of reference. Immediately after, TPA was applied on both faces of the right ear. The left ear received only acetone as a control. After 4 h, the animals were sacrificed, and a disk (6 mm diameter) removed from both ears (treated and untreated) was individually weighed on an analytical scale (Ohaus PioneerTM, Parsippany, NJ, USA). Edema was indicated by the weight increase of the right ear disk over the left.

Histopathology

Tissue samples were preserved in buffered formalin, embedded in paraffin, 5 μm sections were cut and stained with hematoxylin and eosin (H&E). The presence of edema, epidermal hyperplasia/hypertrophy, infiltration of mononuclear and polymorphonuclear cells, connective tissue disruption, and dermal fibrosis was assessed by a blind pathologist using light microscopy (Axio Lab A1, Zeiss, Oberkochen, Germany).
Myeloperoxidase (MPO) assay

MPO activity was measured according to the method described by Bradley et al. (1982) (Bradley et al., 1982). Ear tissue was weighed and homogenized in phosphate-buffered saline (PBS), pH 7.4. The homogenate was centrifuged at 2576g for 30 min at 41ºC. The pellet was again homogenized PBS, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HTAB). This suspension was subjected to cycles of freezing/thawing and brief periods of sonication (15 s) and centrifuged at 2576g for 10 min at 4ºC. Supernatants were mixed with O-dianisidine dihydrochloride (0.067%) and hydrogen peroxide (0.003%), and incubated for 5 min before measuring the OD_{450}. Enzyme activity was expressed as enzyme activity units, where 1 activity unit is defined as the amount of enzyme capable of metabolizing 1 mmol of hydrogen peroxide in 1 min at 25 ºC.

Measurement of tissue cytokine level

In brief, ear tissue was homogenized in T-PER Tissue Protein Extraction Reagen with protease inhibitor cocktail (Roche), at 4°C using TissueRuptor® (Qiagen, Haan, Germany). Samples were centrifuged at 12.000 rpm at 4°C. The levels of IL-1β, IL-6, and TNF-α were quantified on the obtained supernatants with commercial enzymelinked immunosorbent assay [ELISA] kits [eBioscience], following the manufacturer's protocol. The results were expressed as pg or ng cytokine per milligram of protein, which was quantified by the Bradford method, using a standard commercial kit [Biorad 500-0206].

Measurement of tissue PGE2 level

In brief, ear tissue was homogenized in T-PER Tissue Protein Extraction Reagen with protease inhibitor cocktail (Roche) and indomethacin at 4°C using TissueRuptor® (Qiagen, Haan, Germany). Samples were centrifuged at 12.000 rpm at 4°C. The levels of PGE2 were

quantified on the obtained supernatants with commercial enzymelinked immunosorbent assay [ELISA] kits [eBioscience], following the manufacturer's protocol.

Cellular ROS determination by DCFH-DA

Cell culture

Murine macrophage RAW cell lines (ATCC® TIB-71™, Rockville, MD) were maintained in the DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere at 37°C.

Assessment of cell viability

The toxicity of the total ethanolic extract obtained from the leaves of *Mammea americana* on RAW 264.7 macrophages was evaluated using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric method (Scudiero et al., 1988). RAW 264.7 macrophages were seeded in sterile 96-well plates (2×10^4 cells/well) and incubated at 37°C for 48 hours, then the medium was removed, and cells were washed with phosphate buffer saline (PBS) and treated for 30 minutes with different concentrations of the extract of *Mammea americana*, after which, they were activated with LPS (1 µg/ml) and incubated again for 24 hours at 37°C. Following exposure, the cell culture medium was discarded and 100 µl of MTT solution (0.25 mg/ml) was added to each well of the plate. The plates were incubated at 37ºC for 4 hours in the CO_2 incubator. Finally, 100 µl of DMSO was added to dissolve the formazan crystals, and the absorbance was measured at 550 nm by a Multiskan EX microplate reader (Thermo Scientific, Waltham, MA). In each trial, a group of cells not exposed to the extracts was included as the negative control and a group exposed to Triton X-100 (20%) as control of maximum toxicity. The viability percentages were calculated, considering the negative control group as 100% viability**.**

DCFH assay

The formation of intracellular ROS was determined using the oxidation-sensitive dye DCFH-DA according to the protocol described by Park *et al.* (2017) (Park et al., 2017), with some modifications. For that, macrophages RAW 264.7 (10×10^4 cells/well) were incubated in 96-well sterile black plates. After 24 hours of incubation, the cells were washed twice with sterile PBS to remove nonadherent cells. The remaining cells were incubated for 30 minutes with different concentrations of the total ethanolic extract obtained from the leaves of *Mammea americana* and activated with LPS (1 µg/ ml). After 24 hours of incubation, the cells were washed three times with sterile PBS, stained with DCFH-DA (20 µM) in PBS, and incubated for 30 minutes at 37°C in the dark. Fluorescence intensity was measured in a 96-well Fluoroskan Ascent plate reader (Thermo Scientific, Waltham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Quercetin was used as the positive control.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M) of at least two independent experiments. IC_{50} and LC_{50} were calculated employing non-linear regression and expressed as mean and its 95% confidence interval. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's and Tukey's post hoc tests. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The species *Mammea americana* is widely used in Caribbean traditional folk for its important insecticidal action on a wide range of insects. For the treatments of various pathologies such as skin diseases, fever and inflammation can be used all the plant, with differences in the medicinal preparations according to the country (Lemus et al., 2021). Additionally, several studies focused on the pharmacological activities of *Mammea americana* have shown evidence on the potential Insecticidal activity (Crombie et al., 1972; Sievers et al., 1949), trypanocidal activity (Reyes-Chilpa et al., 2008), anti-cancer properties (Du et al., 2010; Yang et al., 2005), antibacterial effects (Herrera Herrera et al., 2014), antiviral effects (Gómez-Calderón et al., 2017), and anti-ulcerogenic effects (Toma et al., 2005). However, as far as we know, there is research on the anti-inflammatory potential of this plant species at the in vitro level, but there are no published studies that confirm whether this biological activity is maintained in vivo. For this reason, we decided to focus this research work on evaluating the anti-inflammatory potential of the *Mammea americana* species, in the TPA-induced mouse ear edema model, being an important contribution to the knowledge of the biological activities that this species can offer.

An extract from the leaves of *Mammea americana* was obtained, and a phytochemical study was carried out, which revealed the presence of coumarins, tannins, cardiac glycosides, phenolic compounds, flavonoids, saponins, and triterpenes/steroids. These results correspond to those reported in other investigations on the phytochemical composition of this plant species (Lemus et al., 2021). For the evaluation of the anti-inflammatory potential of the extract of leaves of *Mammea americana*, the assay of edema induced by the irritating agent TPA was used. Figure 2.1A shows how treatment with 1 mg of *Mammea americana* extract significantly reduces the weight of the punches, indicating a decrease in edema in atrial tissue. The extract of *Mammea americana* also at both doses used (0.5 and 1 mg) was able to decrease the activity of the enzyme myeloperoxidase (MPO) in the atrial tissue, an enzyme widely distributed in the body, however, its main source is neutrophils, so the measurement of this enzymatic activity has been considered a sensitive quantitative marker of neutrophil chemotaxis and infiltration in the inflammatory process (Figure 2.1B). The anti-edema effect of the extract from the leaves of *Mammea americana* was confirmed by histological examination of the ear tissue. The extract of *Mammea americana* reduced the disruption of tissue structure, immune cell infiltration, hyperplasia and hypertrophy of dermis, and

epidermis; induced by the irritating agent TPA, with similar effect to the produced by indomethacin (Figure 2.1C).

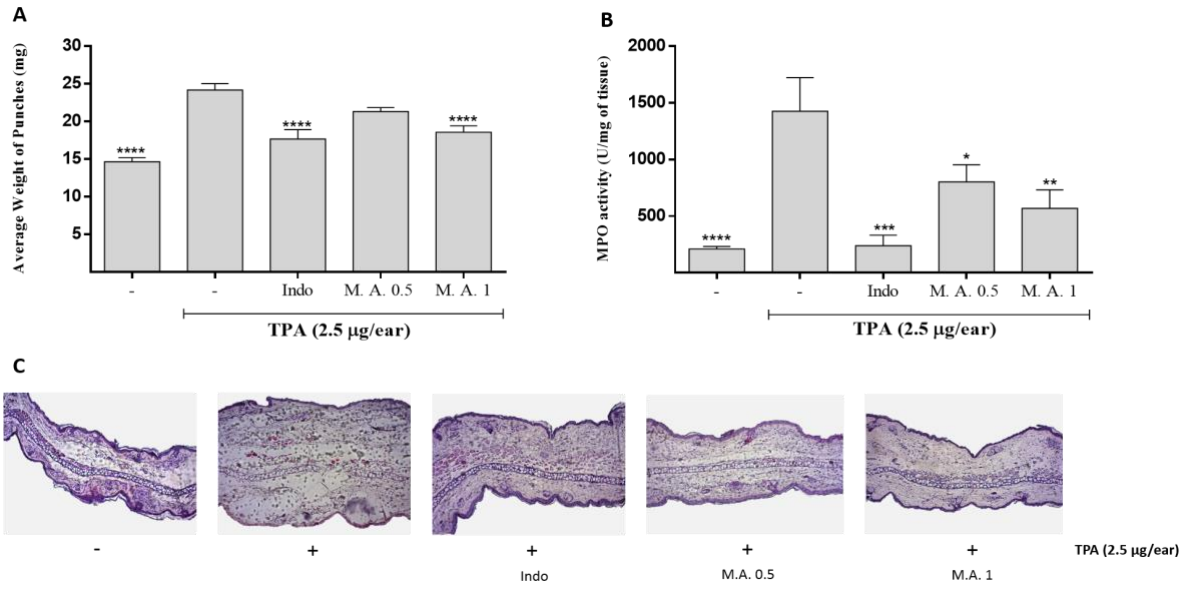

Figure 2.1: Inhibitory effects of topical application of the total ethanolic extract of *Mammea Americana* leaves on TPA-induced edema of mice ears. **A**: Ear of mouse were topically treated with 20 µL of acetone (vehicle control) or extract of *Mammea Americana* leaves (0.5 and 1 mg/ear) 5 minutes prior to application of TPA (2.5µg/ear). The mice were sacrificed 4 h after TPA treatment. Ear punches (6 mm in diameter) were taken immediately and weighed. **B**: Tissue lysates were prepared from ear biopsies and analyzed for MPO activity. **C**: to examine histological ear tissue sections were prepared and stained with hematoxylin and eosin (H&E). Results are expressed as mean ± SEM. (n = 7) (*p < 0.05, , **p < 0.01, ***p < 0.001, ****p < 0.0001 ANOVA, statistically significant vs TPA group).

The irritant agent TPA activates the protein kinase C (PKC), with the consequent increase in reactive oxygen species (ROS), which play an important role in the activation of the MAPK pathway, which regulates the activation of several important transcription factors such as NF-kB and AP1, involved in the production of a wide variety of inflammatory mediators such as IL-1β, IL-6, TNF- α and PGE2 (Noh et al., 2015). This work sought to identify if the anti-edema effect shown by the extract of *Mammea americana* could be related to a decrease in the levels of PGE2, pro-inflammatory cytokines and ROS; taking into account that in a previous study on the anti-inflammatory potential of this species it was found that the extract of *Mammea americana* inhibited the levels of these cytokines in macrophages raw 264.7, an effect that was also evidenced *in vivo*. The total extract of the leaves of *Mammea americana* was able to decrease in a concentration-dependent manner the levels of the cytokines IL-1β, IL-6 and TNF- α in the mice ears treated with TPA, although only the levels of IL-1β and IL-6 decreased significantly. On the other hand, *Mammea americana* extract had no effect on PGE2 levels (Figure 2.2).

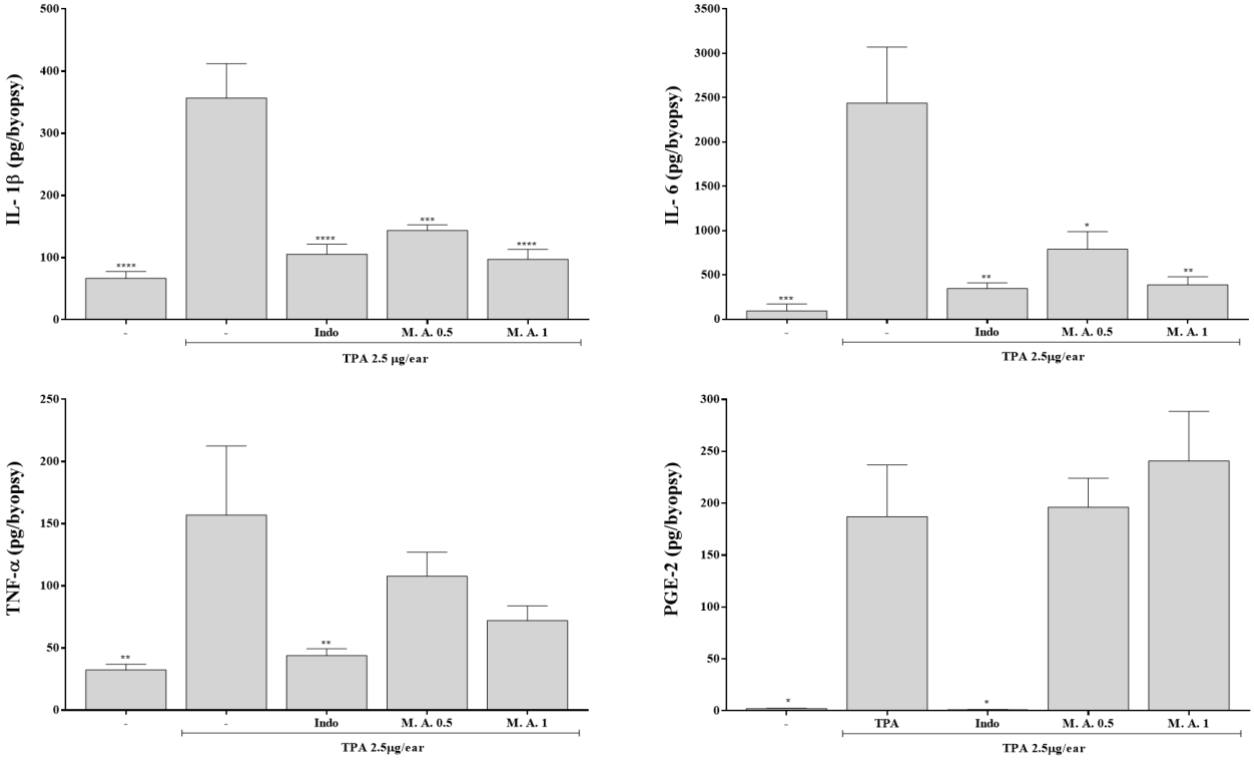

Figure 2.2: Inhibitory effect of the total ethanolic extract of *Mammea americana* leaves on the production of IL-1β, IL-6, TNF-α and PGE2 in TPA-treated mouse ears. Ear of mouse were topically treated with 20 µL of acetone (vehicle control) or extract of *Mammea Americana* leaves (0.5 and 1 mg/ear) 5 minutes prior to application of TPA (2.5µg/ear). The mice were sacrificed 4 h after TPA treatment. Ear punches (6 mm in diameter) were taken immediately and weighed. Tissue lysates were prepared from ear biopsies and analyzed for the levels of cytokines by ELISA. Results are expressed as mean ± SEM. (n = 7) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 ANOVA, statistically significant vs TPA group).

Mammea americana leaves contain a significant amount of phenolic compounds and the total ethanol extract of *Mammea americana* leaves has shown significant scavenging activity for free radicals DPPH and ABTS (Jenny P. et al., 2021), this antioxidant potential shown by the extract of leaves of *Mammea americana* is maintained in a biological matrix, since this extract was able to inhibit the increase in ROS levels in LPS-stimulated RAW 264.7 macrophages, above basal levels, showing the same antioxidant potential as quercetin, a flavonoid used as a positive control (Figure 2.3). The extracts of *Mammea americana* showed no toxicity on RAW 264.7 macrophages at 25 and 50 µg/mL (data not showed). The simultaneous suppression of IL-1β, IL-6, TNF-α, and ROS by the extract of leaves of *Mammea americana* shows the important potential that this plant species can have in the treatment of inflammatory processes.

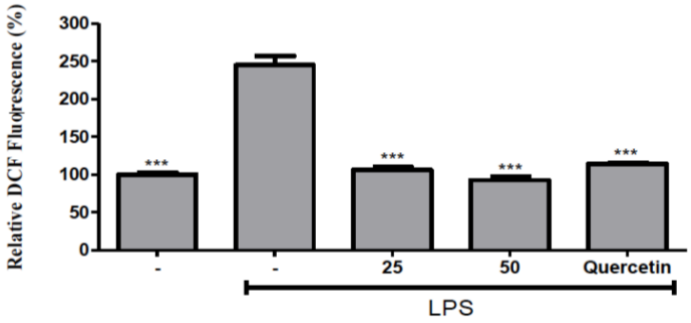

Figure 2.3: Effect of the total ethanolic extract of *Mammea americana* leaves on production of intracellular ROS in LPS-stimulated RAW 264.7 macrophages (1 µg /mL) for 24 h. Reactive oxygen species are expressed as fold increase of 2′,7′-dichlorofluorescein's fluorescence over LPS-untreated controls. To cell viability and relative DCF fluorescence the results represent the mean ± standard error of the mean. (***p<0,001 statistically significant compared with untreated group and LPS treated group, respectively).

CONCLUSIONS

Our results show the anti-inflammatory potential of the species *Mammea americana.* The total ethanolic extract of *Mammea americana* leaves significantly reducing the edema, neutrophil infiltration and the production of the IL-6, IL-1β, and ROS inflammatory mediators in a TPA-induced mice ear edema assay. To our knowledge, this is the first scientific report of the anti-inflammatory potential of this plant species, in an *in vivo* model.

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PRODUCTO III:

A SCREENING OF PLANTS USED IN COLOMBIAN TRADITIONAL MEDICINE REVEALED THE ANTI-INFLAMMATORY POTENTIAL OF *PHYSALIS ANGULATA* CALYCES

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ABSTRACT

The use of natural products by communities from the Colombian Caribbean region to treat health issues, together with biodiversity and geographical features, constitute a great scenery to develop new therapies based on ethnopharmacological heritage. Here, we investigated the anti-inflammatory potential of 10 commonly used plants in Colombian folk medicine, evaluating their effect on nitric oxide (NO) production by LPS-stimulated RAW 264.7 macrophages. The most active plant was evaluated *in vivo* using 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced mouse ear edema, along with its effect on the production of pro-inflammatory mediators *in vitro*. The extract of *Physalis angulata* L. calyces showed the highest activity. This extract was fractionated and its dichloromethane fraction (DF) was the most active *in vitro*, inhibiting the production of NO, prostaglandin E2 (PGE2), interleukin (IL)-1b, IL-6, tumor necrosis factor (TNF)- α and monocyte chemotactic protein (MCP)-1 (CCL2). *In vivo,* DF showed a significant inhibition of ear edema and myeloperoxidase (MPO) activity, with evident reduction of the leukocyte infiltration into tissue. Our results support the ethnopharmacological use of the selected plants in folk medicine. *P. angulata* dichloromethane fraction represents a promising source of pharmacological compounds with great potential therapeutic use to treat inflammatory illness.

Key words: Folk medicine, Anti-inflammatory, *Physalis angulata*, Nitric oxide, Macrophages.

INTRODUCTION

Inflammatory mediators are key signaling molecules implicated in the activation and regulation of inflammatory processes and are produced by various hematopoietic and non-hematopoietic cell types (Harden et al., 2013; Sofroniew, 2013). One of the most important mediators of the immune response is the nitric oxide (NO) radical produced by the inducible nitric oxide synthase (iNOS). This small molecule is not only essential for host innate immune response but also has a pivotal physiological role in the regulation of vasodilatation, neuronal transmission, cardiac contraction, stem cell differentiation and proliferation (Lei et al., 2013). However, when iNOS activity is aberrant it has detrimental consequences and promotes multiple human diseases (Pautz et al., 2010). Thus, NO is a fundamental molecule for inflammation development and resolution. Given the simplicity of the NO measure through he Griess reaction, the employment of activated immune cells (i.e. macrophages) has been established as a fast, economic and high-throughput alternative, not only to measure the antiinflammatory potential of a molecule, but as a great criteria for bioassay-guided fractionation (Ye et al., 2014).

Chronic anti-inflammatory therapy carries several unwanted secondary effects and poses burden on the patients, reducing therapeutic adherence and compromising the effectiveness of the treatment (Tabas and Glass, 2013). To address this, drug Discovery research has focused on synthetic and computational methods, leaving aside bioprospecting as an alternative to identify new chemical entities (Macilwain, 1998; Rishton, 2008). Despite this, medicinal plants have been a major source of bioactive molecules for years and continue to be an essential alternative when it comes to health care in many places around the world, especially the rural areas of developing countries, where socio-economic factors difficult the access to modern drugs and health care systems (Cragg and Newman, 2013). In countries like Colombia, with more than 10% of the global biodiversity, folk medicine is used as a primary health care, by communities with knowledge and regular Access to medicinal plants (Gómez-Estrada et al., 2011). In this context, ethnopharmacological research and bio-guided isolation of active secondary metabolites remain a crucial strategy in the Discovery of safer and more effective drugs (Mishra and Tiwari, 2011).

The Colombian Caribbean region has unique geographical and topographical features that had molded not only an extremely diverse flora and fauna, but also a great cultural diversity based on a wide ethnopharmacological knowledge centered on natural resources (Gómez-Estrada et al., 2011). Despite Colombian government efforts to protect natural biodiversity and traditions related to medicinal plants, factors like discrimination to minorities, forced migrations and lack of policies, have affected the preservation of unique traditions to Colombian culture (Gómez-Estrada et al., 2011; Regalado, 2013; Velásquez-Tibatá et al., 2013).

In an effort to document and increase the attention to biodiversity conservation and ethnopharmacology, our research group started a bioprospecting program based on traditional knowledge of anti-inflammatory plants from the Colombia Caribbean region. The aim of this work was to assess the effect of 10 plants frequently used in the folk medicine to treat inflammation related diseases and that have been previously reported for its traditional uses (Table 3.1.). Anti-inflammatory potential was assessed using the NO production in lipopolysaccharide (LPS)-stimulated macrophages, the most active plant was fractionated and evaluated using 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced mouse ear edema, measuring histopathological parameters and myeloperoxidase activity (MPO). Complementarily, the most active plant was further studied regarding its effects on the production of prostaglandin (PG)E2, cytokines and chemokines by LPS-stimulated macrophages. Additionally, the free radical scavenging potential was evaluated using NO, ABTS and DPPH scavenging assays.

Table 3.1: List of selected medicinal plant species employed in Colombian Caribbean Region to treat inflammatory related diseases including their ethno-pharmacological characteristics

Scientific name	Local name	ethnopharmacological use	Part used	Mode of preparation/administration route	Reference
Calotropis procera (Aiton) Dryand. (Apocynaceae)	Algodón de seda	Headache Rheumatism	Roots Leaves	Exudate/Oral Cataplasm/Topic	Lal and Yadav (1983) Kumar Vijay et al. (2011)
Ceratopteris pteridoides (Hook.) Hieron. (Pteridaceae)	Cola de caballo	Respiratory Conditions Diuretic and Cholelithiasic	Leaves	Infusion/Oral	Alviz et al. (2013) Beltrán Villanueva et al. (2013)
Cordia alba (Jacq.) Roem, & Schult. (Boraginaceae)	Sauce sabanero	Bronchitis	Bark, Buds	Oil Decoction, Infusion/Oral	Waizel-Bucay (2005)
Croton malambo H.Karst. (Euphorbiaceae)	Croton	Rheumatism Pain Inflammation	Bark	Infusion/Oral	Salatino et al. (2007) Suárez et al. (2003)
Cryptostegia grandiflora Roxb. ex R. Br. (Apocynaceae)	Flor de muerto	Inflammation Infection	Leaves	Fresh plant in maceration/Cataplasm	Castro et al. (2014) Rivera et al. (2015)
Dracontium dubium Kunth (Araceae)	Chupadera	Snakebite Inflammation	Root	Poultice/Topic	Lévi-Strauss (1952)
Maclura tinctoria (L.) D. Don. ex Steud. (Moraceae)	Palomora	Inflammation Toothache Rheumatism	Bark	Latex Drops/Topic Decoction, /Oral	Beltrán Villanueva et al. (2013)
Merremia umbellata (L.) Hallier f. (Convolvulaceae)	Juan de Dios	Inflammation and Infections Antiulcer Rheumatism	Leaves Whole Plant	Decoction/Oral	Castro-Guerrero et al. (2013) Rivera et al. (2015)
Physalis angulata L. (Solanaceae)	Topo toropo	Inflammation and Infections Abdominal Cramps Asthma, Dermatitis, Rheumatism	Calyces Aerial Parts Whole Plant	Poultice/Topic Infusion/Oral Infusion/Oral	Rivera et al. (2015) Soares et al. (2003)
Tabebuia rosea (Bertol.) Bertero ex A.DC. (Bignoniaceae)	Palo de rosa	Inflammation Fever	Bark Stem, Bark	Decoction/Oral	Gómez-Estrada et al. (2011)

MATERIALS AND METHODS

Chemicals

12-O-tetradecanoyl-phorbol-13-acetate (TPA), indomethacin, ethylenediaminetetraacetic acid (EDTA), O-dianisidine, dulbecco's modified eagle medium (DMEM), penicillin–streptomycin, trypan blue, lipopolysaccharide (LPS), N-([3-(aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride (1400W), sodium nitrite, N-(1,1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, hematoxylin-eosin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dichloromethane, dimethylsulfoxide, ethanol, and petroleum ether were obtained from Merck (Kenilworth, NJ, USA) or Carlo Erba (Val-de-Reuil, France). Hexadecyltrimethylammonium (HTAB), hydrogen peroxide (H_2O_2), bromide of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) from Calbiochem_ (San Diego, CA, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Gaithersburg, MD, USA). All other chemicals and reagents used were of analytical grade.

Plant material and preparation of the extracts

The plant material was collected in the Caribbean coast of Colombia and plants were identified by a botanist at the Herbarium of the University of Antioquia (HUA, Medellin, Antioquia, Colombia) and the Botanical Garden Guillermo Piñeres (JBC, Túrbaco, Bolivar, Colombia) where voucher specimens were deposited (Table 3.2).

Except for *Dracontium dubium*, all plant material were dried at room temperature, powdered and extracted by maceration with ethanol at room temperature until exhaustion. The crude ethanolic extract was filtered and the solvent evaporated under reduced pressure at 40 ± 5ºC using a rotary evaporator (Heidolph VV 2000, Heidolph, Kelheim, Germany). Tubers of *D. dubium* were washed, peeled and crushed in a minimum amount of water, then lyophilized (FreeZone_ Dry 2.5, Labconco, Kansas, MO, USA). The powder obtained was finally extracted by maceration as described before.

Physalis angulata extract was fractionated through liquid-liquid partition with petroleum ether, dichloromethane and methanol-water (9:1) to obtain a petroleum ether (EF), dichloromethane (DF) and methanol fraction (MF).

Test extracts/fractions were dissolved in dimethyl sulfoxide (DMSO) or ethanol to obtain a stock solution, and stored at -20ºC. The final percentage of vehicle was adjusted to 0.1% (v/v) in cell culture experiments.

Phytochemical screening, flavonoids and polyphenols quantification

The phytochemical screening for presence of terpenes, steroids, quinones, glycosides, alkaloids and tannins was performed as reported by Herrera et al. (2014). Polyphenols were quantified using the Folín-Ciocalteu method with some modifications (Del- Toro-Sánchez et al., 2014). Briefly, the sample was mixed with Folín-Ciocalteu reagent (0.1 M), and 10 min later a solution of sodium carbonate (7.5%) was added. After incubating the mixture for 2 h at room temperature (RT), the Optic Density at 760 nm (OD_{760}) was determined using a microplate reader (Multiskan EX Thermo, Waltham, MA, USA). Results are expressed as mg of gallic acid equivalents (GAE) per gram of extract.

Flavonoids were determined by the aluminum trichloride method with some modifications (Adebayo et al., 2015). Samples were mixed with sodium nitrite (1.5%) and stirred for 10 min at RT, aluminum trichloride (3%) was then added and the mixture was now stirred for 5 min, before finally adding sodium hydroxide (1N). After 10 min, the OD_{450} was measured. Results are expressed as mg of catechin equivalents (QE) per gram of extract.

Table 3.2: Plant information: list of evaluated plants, collection sites, vouchers, organs used, extraction solvent and yields.

Scientific name	Collection site	Voucher number	Used part	Extract yields (%)
Calotropis procera	Galerazamba, Bolívar (10°47′52″N 75°15′41″W)	HUA175327	Leaves	9,73
Ceratopteris pteridoides	San Bernardo del Viento, Cordoba (9°21′18″N 75°57′16″W)	HUA166134	Whole Plant	6,79
Cordia alba	Pueblo Nuevo, Bolívar (10°44′26″N 75°15′43″W)	HUA175329	Flowers	11,59
Croton malambo	Galerazamba, Bolívar (10°47′22″N 75°15′36″W)	JBC 12,008	Bark	8,82
Cryptostegia grandiflora	Pueblo Nuevo, Bolívar (10°44′33″N 75°15′28″W)	HUA175331	Leaves	6,92
Dracontium dubium	La Unión, Sucre (8°51′37″N 75°16′48″W)	HUA 189,121	Root	N.D.
Maclura tinctoria	Pueblo Nuevo, Bolívar (10°44′42″N 75°15′48″W)	JBC 12,013	Bark	5,33
Merremia umbellata	Pueblo Nuevo, Bolívar (10°44′22″N 75°15′35″W)	HUA175331	Leaves	14,44
Physalis angulata	Pueblo Nuevo, Bolívar (10°44′N 75°15′W)	HUA175328	Calyces	16,05
Tabebuia rosea	Túrbaco, Bolívar (10°19′55″N 75°24′51″W)	HUA196871	Bark	9,20

All extracts were obtained by maceration with ethanol 96% and concentrated in a rotaryevaporator. The yields were calculated as: [Dry Concentrated Extract (g)/Dry Material (g)] × 100. N.D.: Not determined.

In vitro anti-inflammatory activity

Cell culture

RAW 264.7 (TIB-71TM), murine macrophages were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10% FBS and penicillin (10 UI/mL)/streptomycin (10 µg/mL) at 37ºC and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80–90% confluence.

Cell viability

The reduction of MTT to formazan was used to assess the effects of test extracts/fractions on cell viability following the protocol described by Mosmann, 1983. Cells (2 x 10^5 cells/mL) were seeded in 96-well microplates and treated with vehicle (DMSO/ethanol) or extracts/fractions (100–6.25 µg/mL) for 30 min, then stimulated with LPS (10 µg /mL). After 24 h, cells were incubated with MTT solution (0.25 mg/mL), 4 h later the formazan was dissolved in DMSO and the OD_{550} was measured. Triton X-100 (2%) was used as positive control. Percentages of cell survival relative to control group were calculated, as well as the lethal concentration 50% (LC_{50}).

Pro-inflammatory mediators assessment

Cells ($2x10^5$ cells/mL) were seeded in 24-well microplates and treated with vehicle, test extracts/fractions (100–6.25 µg/mL), 1400W (2.50 µg/mL), dexamethasone (7.85 µg/mL), or rofecoxib (6.29 µg/mL) for 30 min, then stimulated with LPS (10 µg/mL). After 24 h, culture supernatants were collected and kept at -20ºC until further analysis.

NO release was determined using the Griess reaction (Green et al., 1982). Equal volumes of supernatants and Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4) were mixed and incubated at RT for 5 min. The OD_{550} was measured and compared with a sodium nitrite standard curve (1–200 µM). Percentage of inhibition was calculated using control cells and the IC_{50} was determined. To corroborate that NO depletion was caused by biological inhibition, instead of a scavenging effect of the extracts, the scavenging activity was measured using the method by Franco et al., 2014.

Production of PGE_2, IL-1β, IL-6, TNF-α and CCL2 was measured using ELISA following the instructions of the manufacturer (eBiosciences, San Diego, CA, USA).

***In vivo* anti-edema activity**

Experimental animals

Female ICR mice (20–25 g) provided by the Instituto Nacional de Salud (Bogotá, Colombia) were allowed to acclimatize for at least two weeks before use and fed standard rodent food and water ad libitum. They were housed in filtered-capped polycarbonate cages and kept in a controlled environment (22±3 ºC and relative humidity: 65 to 75%), under a 12 h light/darkness cycle. All experiments were designed and conducted in accordance with the European Union regulations (CEC council 86/809) and approved by the Ethics Committee of the University of Cartagena (Minutes No. 32 August 4, 2011).

TPA-induced acute ear edema

The edema was induced by topical application of TPA (2.5 μg/ear) in acetone, according to the method described by De Young et al., 1989. Groups (n = 6) were treated on the inner and outer surfaces of the right ear with the acetone dissolved treatment (1 or 0.5 mg in 20 μL of vehicle) or indomethacin (0.5 mg in 20 μL of vehicle), as reference drug. Immediately after, TPA was applied on both faces of the right ear. The left ear received only acetone, as a control. After 4 h, the animals were sacrificed, and a disk (6 mm diameter) removed from both ears (treated and untreated) was individually weighed on an analytical scale (Ohaus Pioneer^{TM}, Parsippany, NJ, USA). Edema was indicated by the weight increase of the right ear disk over the left, and the anti-edema activity was expressed as inhibition percentage of edema.

Histopathology

Tissue samples were preserved in buffered formalin, embedded in paraffin, 5 μm sections were cut and stained with hematoxylin and eosin (H&E). The presence of edema, epidermal hyperplasia/hypertrophy, infiltration of mononuclear and polymorphonuclear cells, connective tissue disruption and dermal fibrosis was assessed by a blind pathologist using light microscopy (Axio Lab A1, Zeiss, Oberkochen, Germany). Parameters were scored from 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe).

Myeloperoxidase (MPO) assay

MPO activity was measured according to the method described by Bradley et al. (1982). Ear tissue was weighed and homogenized in phosphate-buffered saline (PBS), pH 7.4. The homogenate was centrifuged at 2576g for 30 min at 4 ºC. The pellet was again homogenized PBS, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (HTAB). This suspension was subjected to cycles of freezing/thawing and brief periods of sonication (15 s) and centrifuged at 2576g for 10 min at 4ºC. Supernatants were mixed with O-dianisidine dihydrochloride (0.067%) and hydrogen peroxide (0.003%), and incubated for 5 min before measuring the OD_{450}. Enzyme activity was expressed as enzyme activity units, where 1 activity unit is defined as the amount of enzyme capable of metabolizing 1 mol of hydrogen peroxide in 1 min at 25 ºC.

Radical scavenging activity

The ABTS-scavenging effect was determined using the method described by Re *et al*. (1999), with modifications. ABTS (3.5 mM; in ethanol) and $K_2S_2O_8$ (1.25 mM) were mixed with ascorbic acid (positive control) or test extracts/fractions and incubated at RT for 5 min. The scavenging of the ABTS radical was determined by measuring the OD_{405}. The DPPH-

scavenging effect was determined using the method described by Brand-Williams et al. (1995), with some modifications. In brief, DPPH (100 μg/mL), was mixed with ascorbic acid or test extracts/fractions, and then incubated at RT for 30 min. The scavenging of the DPPH radical was determined by measuring the OD_{550}. Vehicles were used as negative controls.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (S.E.M) of at least two independent experiments. IC_{50} and LC_{50} were calculated employing non-linear regression and expressed as mean and its 95% confidence interval. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's and Tukey's post hoc tests. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Ethnopharmacological studies allow not only to document traditional knowledge of plants, but to engage policy-makers in designing appropriate strategies for the conservation of biodiversity and cultural knowledge related to medicinal plants. We contribute to this endeavor through our bioprospecting program, focused on isolation of bioactive compounds or therapeutically useful extracts/fractions from plants of the Colombian Caribbean region. In the first part of our work the anti-inflammatory potential of 10 plants traditionally used to alleviate inflammation (Tables 2.1 and 2.2) were evaluated based on their capacity to inhibit NO production by LPS-stimulated RAW 264.7 macrophages. In the second part, we focused on the fractionation and evaluation of the most active extract, as well as some insights into the mechanism of action.

Effect of the plant extracts on the NO production by LPS-stimulated RAW 264.7 macrophages

Stimulation of RAW 264.7 macrophages with LPS (10 μg/mL) elicited a significant accumulation of nitrite in the medium (41.36 ± 0.63 μM). As can be seen in Table 3.3, NO production was reduced by test extracts at different degrees. We employed the NO inhibition rates (IR) to classify plants among four categories: inactive (–), when the IR was below 20%; weakly active (+), when between 20 and 40%; moderately active (++), with IRs between 40 and 60%; and highly active (+++) when the IR was higher than 60%. Extracts were evaluated at non-toxic concentrations (cell viability above 85%) as determined by the MTT assay (Table 3.3), and did not show any significant NO-scavenging effect (data not shown). Therefore, the NO inhibition presented by the extracts is due to an effect on the cellular processes and not an *in vitro* scavenging effect or cytotoxicity.

At the evaluated concentrations, *D. dubium* and *T. rosea* showed the lowest activity with IRs below 10%. Although previous studies demonstrated the anti-inflammatory activity of *T. rosea* (Franco Ospina et al., 2013) and the anti-edema properties of *Dracontium species* (Gomes et al., 2010), our results are in agreement with several studies that demonstrated that NO production is not inhibited by extracts from plants belonging to the *Tabebuia* genus at concentrations below 200 μg/mL (Byeon et al., 2008). This suggests that the inhibition of NO production might not be a decisive target involved in the effect of these plants. On the other hand, the plants classified as weakly active, *C. pteridoides* and *C. alba*; have no reports of anti-inflammatory activity. However, there are reports of compounds isolated from other plants of the *Cordia* genus with this biological activity (Al-Musayeib et al., 2011; Geller et al., 2010). In this sense, their potential as treatment for inflammation could not be denied, as we employed a stringent top test concentration (100 μg/mL) and focused only on NO production.

To our knowledge, the anti-inflammatory effect of *M. tinctoria* (moderately active) has not been previously reported, positioning our study as the first precedent of the potential of this plant to treat inflammation. On the other hand, the results for *M. umbellata*, also classified as moderately active, are consistent with our previous report that determined its effectiveness reducing the edema and MPO activity in the TPA-induced mouse ear edema model; and supporting the traditional use given to this species (Castro- Guerrero et al., 2013). Likewise, *C. procera* leaves have also been reported to reduce edema and itching on different *in vivo* models (Saba et al., 2013), our results corroborate these findings and place the inhibition of the NO production as a possible target, partially responsible for *M. umbellata* and *C. procera* leaves anti-inflammatory activity.

Table 3.3: Evaluation of the cell viability and NO inhibition potential of extracts obtained from Colombian Caribbean Coast plants on LPS-stimulated RAW 264.7 macrophages.

Plant		Concentration (μg/mL)	% Cell viability	% Nitrite inhibition	Rank
1	*Calotropis procera*	100	$100 \pm 6.70^{**}$	45.11 ± 4.50	++
2	*Ceratopteris pteridoides*	100	$99.10 \pm 1.23^{***}$	33.48 ± 2.29	+
3	*Cordia alba*	100	$100 \pm 8.91^{***}$	33.15 ± 2.69	+
4	*Croton malambo*	50	$86.56 \pm 1.66^{***}$	97.05 ± 2.15	+++
5	*Cryptostegia grandiflora*	100	$98.48 \pm 0.63^{***}$	70.47 ± 0.81	+++
6	*Dracontium dubium*	100	$96.45 \pm 0.65^{***}$	7.23 ± 1.52	–
7	*Maclura tinctoria*	100	$102.11 \pm 1.61^{***}$	41.07 ± 2.12	++
8	*Merremia umbellata*	100	$100 \pm 0.81^{***}$	56.06 ± 1.74	++
9	*Physalis angulata*	25	$93.84 \pm 1.41^{***}$	91.94 ± 1.97	+++
10	*Tabebuia rosea*	100	$96.65 \pm 0.60^{***}$	9.23 ± 1.89	–
11	1400W	2.50	$100 \pm 1.59^{***}$	83.88 ± 1.07	+++

The cell viability was measured using the MTT assay and the $NO^{\cdot}$ inhibition was assessed with the Griess assay as described in Methods. Extracts were classified by their activity as highly active ($IR \geq 60\%$, +++), moderately active ($60\% > IR \geq 40\%$, ++), weakly active ($40\% > IR \geq 20\%$, +), and inactive ($IR \leq 20\%$, –). 1400 W: N-(3-(aminomethyl) benzyl)acetamidine. Each value represents mean ± SEM (n = 8) from at least two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group (One way ANOVA).

The extracts of *C. grandiflora*, *C malambo* and *P. angulata* were catalogued as highly active. *C. grandiflora* extract inhibited NO production by 70%, in accordance with our previous findings that proved its capacity to reduce edema, MPO activity and leukocyte infiltration (Castro et al., 2014). Due to the cytotoxic effect of the *C. malambo* and *P. angulata* extracts, they were evaluated at the lower concentrations. Despite this, both extracts were highly active, with IRs above 90%, making them the most promising extracts in the group. The efficacy of NO inhibition was estimated with the Selectivity Index (SI), defined as LC_{50}/IC_{50}, and used as criteria to choose the best fractionation candidate. As seen in Table 3.4, *C. malambo* and *P. angulata* had the lowest IC_{50} values. Nevertheless, the SI for *P. angulata* (32.15) doubled the SI for *C. malambo* (16.05), suggesting the potential of *P. angulata* as a safer anti-inflammatory agent and a better candidate for fractionation.

The anti-inflammatory activity of *P. angulata* has been widely studied, proving its capacity to reduce inflammation and modulate NO production by extracts from roots (Bastos et al., 2008) and aerial parts (Choi and Hwang, 2003; Sun et al., 2011) as well as by different isolated compounds (Pinto et al., 2010; Soares et al., 2006; Sun et al., 2010). However, our results constitute the first report of anti-inflammatory potential of its calyces. The distinctive calyx of the *Physalis* genus is an important structure formed by sepals as a source of carbohydrates for development and protection of the fruit against environmental threats (Puente et al., 2011). Hence it represents a rich source of novel bioactive metabolites with a vast pharmacological potential, this is supported by other recent reports (Franco et al., 2014; Takimoto et al., 2014).

Assessment of the anti-inflammatory effect of the primary fractions obtained from *P. angulata* calyces

The total ethanolic extract of the calyces of *P. angulata* was fractionated through liquid-liquid partition, obtaining three primary fractions, a petroleum ether (EF), dichloromethane (DF) and methanol fraction (MF). These fractions were evaluated for its potential to inhibit NO production on RAW 264.7 macrophages.

EF and MF were evaluated both at 75 μg/mL whereas DF was evaluated at 25 μg/mL, in accordance with the MTT cell viability test (data not shown). Despite the difference in concentration, DF had a significantly higher NO IR (97%), compared to EF and MF, with IR below 50% (Table 3.5), indicating that the bioactive metabolites were concentrated in the medium polarity fraction. Moreover, DF displayed a significant concentration-dependent inhibition with IC_{50} of 2.48 (2.23–2.77) μg/mL, showing a comparable activity than the iNOS selective inhibitor 1400W (IC_{50} of 3.72 (2.98–4.57) μg/mL). Moreover, none of the fractions displayed NO scavenging effects (data not shown).

Table 3.4: Assessment of the LC_{50}, IC_{50} (NO), and SI of the extracts with the highest NO inhibition potential among the tested plants group.

Plant		LC_{50} ± confidence intervals (μg/mL)	IC_{50} ± confidence intervals (μg/mL)	SI
1	*Croton malambo*	113.907 (97.537–131.361)	7.097 (6.229–8.199)	16.05
2	*Cryptostegia grandiflora*	>100	43.442 (41.748–45.136)	>2.30
4	*Physalis angulata*	130.628 (114.505–144.937)	4.063 (3.541–4.665)	32.15

The cell viability was measured using the MTT assay and the NO inhibition was assessed with the Griess assay as described in Methods. IC_{50} and LC_{50} were calculated with non-linear regression and expressed as mean and its 95% confidence interval ($n = 8$) from at least two independent experiments.

Table 3.5: Evaluation of the NO inhibition potential of fractions obtained from *Physalis angulata* calyces on LPS-stimulated RAW 264.7.

P. angulata fractions		Concentration (μg/mL)	% Nitrite inhibition	Rank
1	Ether (EF)	75	42.897 ± 1.791	++
2	Dichloromethane (DF)	25	97.079 ± 0.494	+++
3	Methanol (MF)	75	49.232 ± 1.138	+

The cell viability was measured using the MTT assay and the NO inhibition was assessed with the Griess assay. Fractions were classified by their activity as highly active (IR ≥ 60%, +++), moderately active (60%>IR ≥ 40%, ++), weakly active (40%>IR ≥ 20%, +), and inactive (IR ≤ 20%, –). Each value represents mean ± SEM ($n = 8$) from at least two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group (One way ANOVA)

The extract and fractions were also evaluated on the TPA induced ear edema model. TPA is an irritating agent that activates protein kinase C (PKC), increasing the levels of pro-inflammatory mediators such as PGE2, TNF- α, IL-1β, and IL-6; which are accompanied by edema, epidermal hyperplasia and immune cell infiltration (Gábor, 2003; Murakawa et al., 2006). TPA application increased the ear thickness after 4 h (Figure. 3.1A), ear edema was significantly ameliorated by treatment with extract and fractions from *P. angulata* calyces. DF showed a dose-dependent reduction of the edema, with the dose of 1 mg/ear inhibiting up to 60% ($P < 0.001$), an effect comparable to the produced by indomethacin (0.5 mg/ear). None of the tested extract/fractions produced evident local or systemic adverse effects, suggesting that their topical administration is well tolerated.

The neutrophil accumulation is a characteristic feature of TPA induced edema (Sánchez and Moreno, 1999) and is measured indirectly by the increase of MPO activity ($P < 0.001$). As seen in Figure 3.1B, treatment with extract and fractions from *P. angulata* significantly decreased the MPO activity relative to the TPA group ($P < 0.001$). The highest activity was exerted by both MF and DF, which inhibited the enzymatic activity by over 71% at 1 mg/ear ($P < 0.001$). Additionally, DF also showed MPO inhibition at 0.5 mg/ear (39% inhibition $P < 0.001$) indicating a dose-dependent effect.

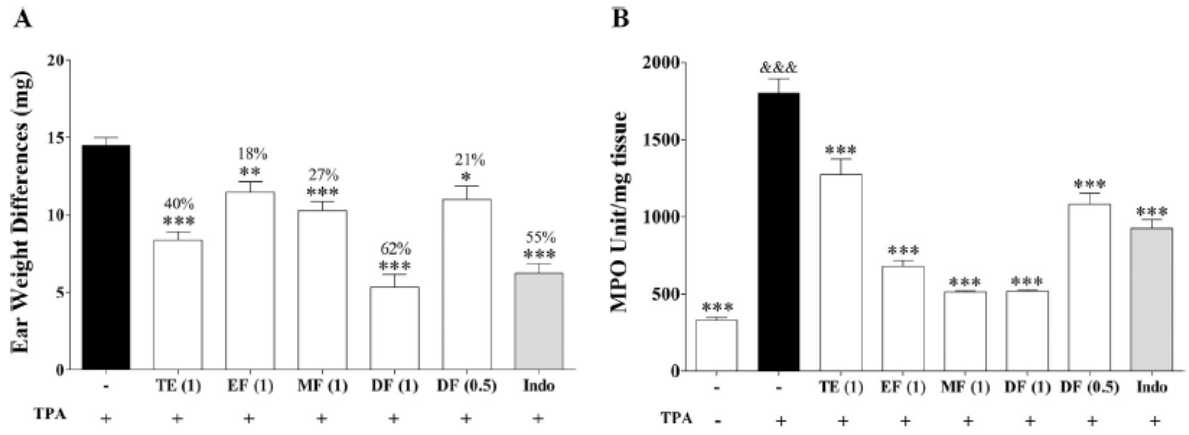

Figure 3.1: *In vivo* evaluation of the anti-inflammatory effect of the extract and fractions obtained from the calyces of *P. angulata* in the TPA-induced mice ear edema. TE (1): Total extract (1 mg/ear), EF(1): Ether fraction (1 mg/ear), MF(1): Methanol fraction (1 mg/ear), DF(1): Dichloromethane fraction (1 mg/ear), DF(0,5): Dichloromethane fraction (0,5 mg/ear), Indo: Indomethacin. (A): Ear weight difference (mg) and inflammation inhibition percentage, circular 6 mm diameter sections of both treated and non-treated ear were weighted. (B): Enzymatic activity measured in ear tissue homogenized expressed as MPO units/mg tissue. Each value represents mean ± S.E.M. (n = 6) from at least two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with TPA group (One way ANOVA). &&&P < 0,001 compared with Control group (One way ANOVA).

The anti-edema effect of the test extract and fractions was confirmed by histological examination of the ear tissue (Figure 3.2). The edema on the TPA group (score: 16), was accompanied by increased ear thickness, disruption of tissue structure, immune cell infiltration, hyperplasia and hypertrophy of dermis, and epidermis (Figure 3.2B). Administration of extract and fractions reduced the tissue damage induced by TPA (Figure 3.2D–H), DF showed the highest antiedema activity, restoring tissue architecture and diminishing the edema, cell infiltration which is reflected in the reduced lower histological score (Score: 4) (Figure 3.2G and H) similar to the effect produced by indomethacin with a histological score of 2 (Figure 3.2C).

Taken together, observations at macroscopic and histopathological level reveal that DF has the highest anti-inflammatory potential and represents a prominent source of bioactive metabolites. These *in vivo* results support the use of the NO determination by the Griess assay as a suitable method for a bio-guided fractionation of potentially anti-inflammatory plant extracts, in accordance with Dirsch et al. (1998).

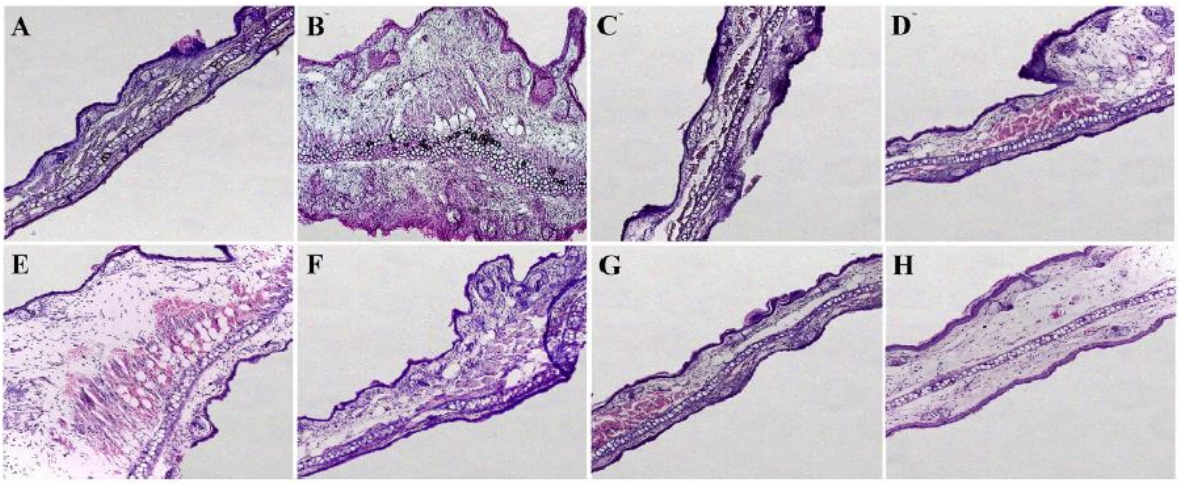

Figure 3.2: Histology samples from the TPA-induced ear edema test, ear tissue was dehydrated, embedded in paraffin and stained with Hematoxylin-Eosin. All pictures were taken in an Axio Lab A1 microscope (Zeiss, Oberkochen, Germany) and correspond to the 10X zoom. (A) Control group, (B) TPA group, (C) Indomethacin (0,5 mg/ear), (D) TE (1 mg/ear), (E) EF (1 mg/ear), (F) MF (1 mg/ear), (G) DF (1 mg/ear) and (H) DF (0,5 mg/ear).

1.2.4.3. Effect of DF on the production of pro-inflammatory mediators by LPS-stimulated RAW 264.7 macrophages

Macrophages are critical effectors and regulators of inflammation and the immune response. When stimulated with LPS, macrophages undergo classical M1 activation through activation of Toll-like receptor 4 (TLR4) (Kayagaki et al., 2013). TLR4 modulation by LPS promotes activation effector functions, NO production and reactive oxygen intermediates (ROI), PGE_2, cytokines (IFN-β, IL-1β, IL-6, IL-12, and TNF-α) and chemokines like the monocyte chemotactic protein-1 (MCP-1, CCL2), as well as other important mediators of the inflammatory response (Bode et al., 2012). M1 macrophages and high NO levels positively correlate with many inflammatory diseases (Gupta et al., 2014). CCL2 provokes mast cell, eosinophil, and macrophage recruitment and activation in rat skin (Sun et al., 2013) and has a critical role in the onset of inflammation and fibrosis (Yamamoto, 2008).

Considering the results showed by DF *in vivo*, we evaluated its effect on pro-inflammatory mediators using LPS-stimulated RAW 264.7 macrophages. As shown in Figure 3.3A–F, high concentration of NO, PGE_2, IL-1β, IL-6, TNF-α, and CCL2 was observed after LPS stimulation, with at least a 4-fold increase ($P < 0.001$). In the absence of LPS, incubation with DF did not modify the release of pro-inflammatory mediators (data not shown). DF significantly inhibited the LPS-induced release of all the evaluated proinflammatory mediators in a concentration-dependent manner (Figure 3.3A–F), with IC_{50} values below 20 μg/mL, suggesting a potential immunomodulatory effect.

Collectively, we have demonstrated that calyces of *P. angulata* promote an anti-inflammatory state of macrophages, decreasing the release of pro-inflammatory mediators. Such effects are, consistent with previous *in vitro* and *in vivo* reports of extracts and compounds obtained from other parts of the *P. angulata* plant, which inhibited NO, PGE_2, IL-6, IL-1β, IL-12, and TNF-α production (Bastos et al., 2008; Sun et al., 2010); or increased IL-4 and IL-10 levels (Sun et al., 2011; Yu et al., 2010), distinctive cytokines of M2 macrophages. We hypothesized that the effects of DF could be related with the blockage of the TLR4 signaling pathway or a profile transition from M1 to M2 macrophages (Martinez and Gordon, 2014), whichever the case might be, further research is needed to determine the actual mechanisms.

Compounds isolated from the calyces of species of *Physalis* genus as physalins (Physalis alkekengi L.), withanolides (Physalis pruinosa L.) and sucrose esters (Physalis peruviana L.), have also show anti-inflammatory potential by the modulation of stimulated macrophages and expression of NO, PGE_2, IL-6, CCL2 or TNF-α, through the inhibition of NF-κB activation (Franco et al., 2014; Ji et al., 2012; Takimoto et al., 2014). Although, we did not study in detail the chemical composition of DF, the phytochemical screening of the fraction showed presence of glycosides, steroids and terpenes (Table 3.6) and colorimetric quantification revealed a low quantity of polyphenols (8.78 ± 0.165 mg Gallic acid/g extract) whereas flavonoids were not detectable. Due to the low concentration of these pharmacologically important secondary metabolites, we think that the biological effects of the fraction may be more influenced by glycosides as they appear to be the most abundant.

As reactive oxygen species are commonly known as triggers of inflammatory processes and its production is considered a potential target of anti-inflammatory drugs (Debnath et al., 2013), we evaluated the potential scavenging effect of *P. angulata* calyces, using DPPH and ABTS assays. The results showed that the total extract and fractions have no radical scavenging effect (100 μg/mL), suggesting that the effects observed, are not related to antioxidant activity (data not shown).

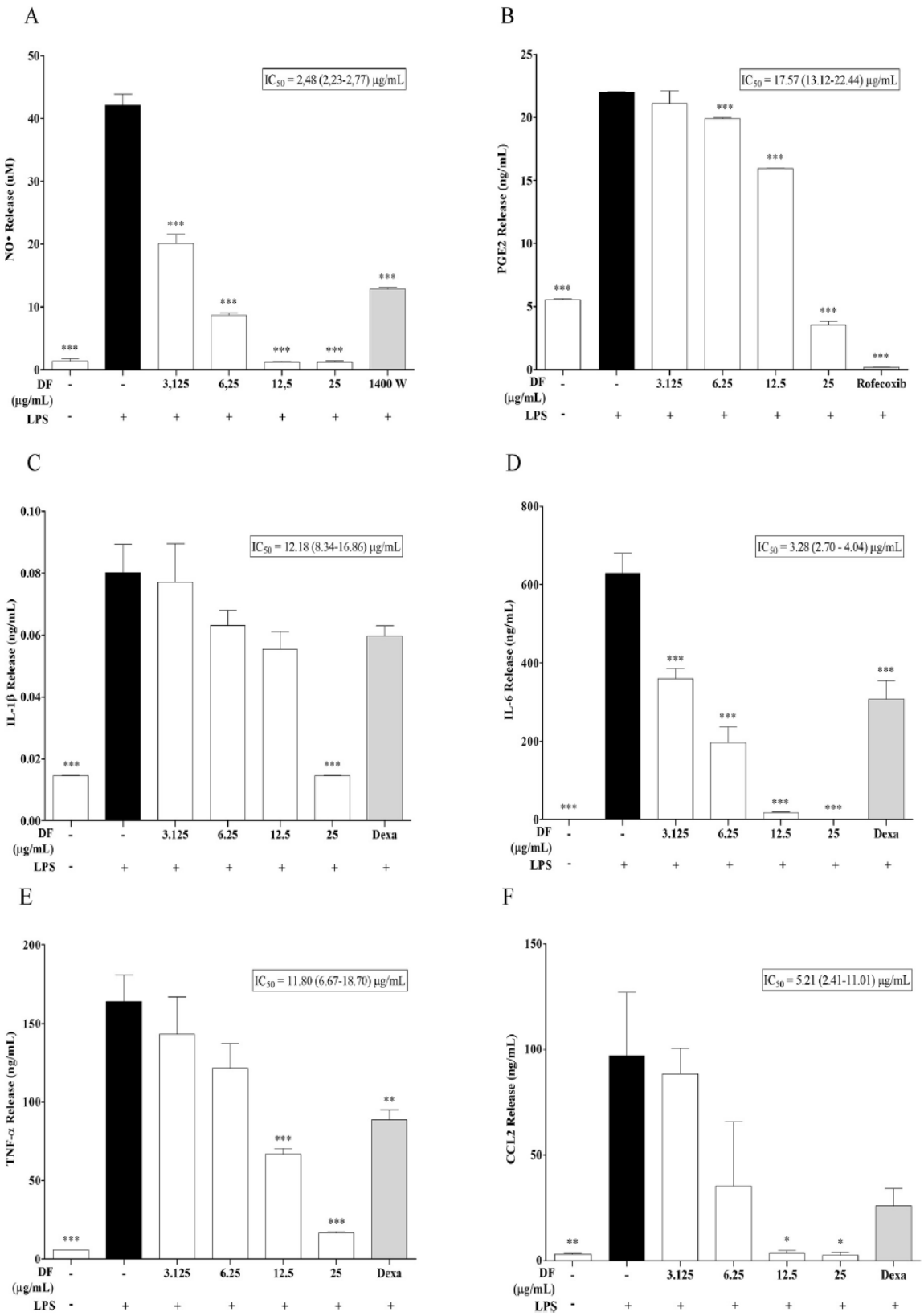

Figure 3.3: Effect of the dichloromethane fraction (DF) on the production of pro-inflammatory mediators by LPS-stimulated RAW 264.7 macrophages. (A): NO_, (B): PGE2, (C): IL- 1 b, (D): IL-6, (E): TNF-a, (F): MCP-1. Cells were incubated for 24 hours after stimulation and treatment with DF. Then supernatants were collected and mediators were measured through ELISA kits, except the NO_ which was measured with the Griess reaction, as described in Methods. Each value represents mean ± SEM (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 compared with LPS group (One way ANOVA).

Table 3.6: Phytochemical characterization of the dichloromethane fraction obtained from the total ethanolic extract of the calyces of *Physalis angulata.*

Metabolite	*P. angulata* DF
Flavonoids	–
Terpenes and/or steroids	++
Quinones	–
Glycosides	+++
Steroidal core	++
Alkaloids	–
Tannins	–

(–) Not detected, (+) Weak presence, (++) Moderate presence and (+++) Strong presence.

Conclusions

In short, our results confirm and support the ethnopharmacological employment of the selected plants in the Colombian folk medicine, constituting the first scientific report of anti-inflammatory potential for *Ceratopteris pteridoides*, *Cordia alba*, and *Maclura tinctoria*. The extract obtained from calyces of *Physalis angulata* had the highest anti-inflammatory potential according to the *in vitro* results, and the dichloromethane fraction inhibited the production of inflammatory mediators (NO, PGE_2, IL-6, IL-1β, TNF-α, and CCL2) by LPS-induced macrophages, as well as reducing the edema, cell infiltration and MPO activity in a TPA-induced mice ear edema assay. To our knowledge, this is the first scientific report of the anti-inflammatory potential of the calyces of *P. angulata*, placing them as promising source of bioactive metabolites. Nevertheless, a bio-guided isolation of bioactive compounds is still necessary.

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PRODUCTO IV:

TOPICAL ANTI-INFLAMMATORY ACTIVITY IN TPA-INDUCED MOUSE EAR EDEMA MODEL AND *IN VITRO* ANTIBACTERIAL PROPERTIES OF *CORDIA ALBA* FLOWERS

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ABSTRACT

Cordia is a genus of trees and bushes widely distributed in warm regions around the world. *Cordia alba* is used in the Colombia Caribbean Coast to treat inflammatory and infectious health issues. The aim of this work was to evaluate the anti-inflammatory and antibacterial activity of the total ethanolic extract obtained from the flowers of *Cordia alba*. The plant material was extracted by maceration with ethanol and the anti-inflammatory effect was evaluated using the *in vivo* model of TPA-induced mouse ear edema and MPO enzyme activity. Additionally, the scavenging activity of the DPPH and ABTS free radicals by the extract was determined. The antibacterial activity was assessed using the broth microdilution method. The total ethanolic extract of *Cordia alba* flowers decreased the edema and the MPO activity in the ear tissue and showed scavenging effects of the DPPH and ABTS free radicals. This extract also presented antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Keywords: Anti-inflammatory, Antibacterial, Ear edema, *Cordia alba.*

INTRODUCTION

For a long time, humanity has used plants for the treatment of diseases, which continue to be one of the most important therapeutic alternatives, estimating that between 70–80% of the world's population relies on traditional medicine to deal with their primary health issues, a tendency acknowledged and approved by the World Health Organization. Natural products have always contributed to the development of modern-day medicine, bringing the discovery of antibiotics, anticancer agents, analgesics and anti-inflammatory compounds (Fabricant and Farnsworth 2001; Kingston 2010; Sen and Samanta 2015). About half of the FDA approved drugs between 1980 and 2010, were natural products, their derivatives, or synthetic mimetics related to natural products (Chen et al. 2015; Ovadje et al. 2015). In this sense, drug

discovery based on the study of medicinal plants remains as an important area in the search for compounds with potential pharmacological activity.

Cordia alba (Jacq.) Roem, and Schult. is native to South and Central America (Cervantes Ceballos et al. 2017). The flowers of this species are widely used in the Caribbean Colombian Coast to treat inflammatory and infectious ailments. Taking into account the use of *Cordia alba* in the folk medicine, this species constitute a promising source of metabolites that could be used for the development of new anti-inflammatory and antibacterial drugs, which are necessary to the medical community, as bacterial resistance to antibiotics has become a public health problem (Fair and Tor 2014) and the anti-inflammatory agents currently used, especially the ones used chronically, have low efficacy and multiple side effects (Joshi et al. 2007; Makris et al. 2010; Harirforoosh et al. 2014). In this work we evaluated the *in vivo* anti-inflammatory effect, *in vitro* free radical scavenger capacity and *in vitro* antibacterial activity of the total ethanolic extract obtained from the flowers of *Cordia alba*.

MATERIALS AND METHODS

Animals

6–7 weeks old, female ICR mice (25–30 g) were provided by the Instituto Nacional de Salud (Bogota-Colombia) and kept at the animal house of the University of Cartagena. They were housed in standard cages under standard conditions (22±3 ºC and 65–75% of relative humidity) with a 12-h dark/light cycle and standard food and water ad libitum. All experiments were designed and conducted in accordance with the guidelines of the Ethics Committee of University of Cartagena (Minute No. 32 of August 4 of 2011) and the European Union regulations (CEC council 86/809).

Collection and identification of plant material

Flowers of *Cordia alba* were collected at Pueblo Nuevo, Bolivar, Colombia (10º44′26″N; 75º15′43″W; elevation 5 m.a.s.l.), identified in the herbarium of the University of Antioquia (Medellin–Colombia) by the biologist Felipe Alfonso Cardona Naranjo and deposited with the identification code HUA 175,329.

Extract preparation

Dried and powdered flowers of *Cordia alba* (176.4 g) were exhaustively extracted with ethanol by maceration at room temperature (25±3 ºC). The extract was then filtered and concentrated using a rotary evaporator (Hei-Vap Value, Heidolph, Germany) under controlled temperature (45–50 ºC) to obtain 20.45 g of total extract.

Phytochemical screening, flavonoids and phenolic compounds quantification

The total extract was screened for the presence of alkaloids, coumarins, flavonoids, cardiotonic glycosides, leucoanthocyanidins, phenolic compounds, quinones, saponins, tannins, and triterpenoids/steroids (Sanabria-Galindo et al. 1997; Thirupathi et al. 2008). Phenolic compounds and flavonoids were quantified using the Folin-Ciocalteu and the aluminum trichloride method respectively (Rivera et al. 2018).

TPA-induced ear edema and myeloperoxidase assay

The anti-inflammatory activity was determined using the *in vivo* murine model of 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced ear edema and MPO activity was determined in the tissue supernatants obtained from the ear edema. Assays were performed as previously described (Rivera et al. 2018). Briefly, a TPA/acetone solution (0.125 μg/μL) was applied topically in both the internal and external side of the mice ear (10 μL/side) to induce inflammation. Immediately after, acetone solutions of *Cordia alba* extract (1000 μg/ear) and indomethacin (500 μg/ear) were applied on the inner and outer surface of the ear. The left ear was treated with just acetone as a control. Four hours after the treatment application, the animals were sacrificed by cervical dislocation and circular sections from both ears (7 mm) were taken and weighted to calculate the edema as weight difference between treated and non-treated ear. The inhibition of inflammation in the treated animals was determined in comparison to the TPA group.

MPO enzyme activity was determined in the tissue supernatants obtained from the ear edema assay, as an indirect measure of neutrophil infiltration. Ear tissue homogenates were prepared with phosphate buffer (pH 7.4). Samples were centrifuged at 10.000 rpm at 4ºC, the pellet obtained was suspended in a solution of HTAB 0.5% and EDTA 0.3% on phosphate buffer (pH 6.0). The homogenate obtained underwent two fast freeze–thaw cycles, sonication for 10 s and lastly it was centrifuged for 10 min at 10.000 rpm at 4ºC. The recovered supernatant was used to evaluate the MPO activity, 50 μL of the supernatant were mixed with 50 μL of O-Dianisidine (0.067%) and 50 μL of hydrogen peroxide (H_2O_2 0.003%). $OD4_{50}$ was determined using a Multiskan EX Thermo. The results are presented as enzymatic activity units, where an activity unit is defined as the amount of enzyme capable of degrading 1 μmol of hydrogen peroxide in a minute at 25 ºC.

DPPH and ABTS radicals scavenging activity

DPPH (1.1-diphenyl-2-picrylhydrazyl) scavenging activity was determined through the method by Brand-Williams et al. (1995) with modifications, 75 μL of the different concentrations of the extract were added to 150 μL of a methanolic solution of DPPH (100 μg/mL). The mix was incubated at room temperature (25 ± 3 ºC) for 30 min; after incubation,

the absence of DPPH radical was measured spectrophotometrically at OD_{517} in a Multiskan EX Thermo. Trolox (50 μM) was used as a positive control and the vehicle as negative control.

Scavenging of the ABTS 2.2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical was determined with the method by Pellegrini et al. (1999), with modifications (Rivera 2018). The ABTS radical was produced by the reaction of ABTS (7 mM) with potassium persulfate (2.45 mM), incubated in the darkness for 16 h at room temperature. The ABTS radical solution was diluted with ethanol until it had an absorbance of 0.8 ± 0.3 at 734 nm, 180 μL of the diluted solution were mixed with 20 μL of different concentrations of the tested extract. This mix was incubated at room temperature (25±3 ºC) for 30 min, and then the absence of ABTS radical was measured spectrophotometrically at OD_{734} in a Multiskan EX Thermo. Trolox (50 μM) was used as a positive control and the vehicle as negative control.

In vitro antibacterial activity

The evaluation of the *C. alba* extract was performed following the protocol by Rivera et al. 2015. A bacterial suspension correspondent to 0.5 of the McFarland scale (1 x 10^8 UFC/mL) was prepared and diluted to obtain a working suspension (5 x10^5 UFC/mL). Equal volumes of the extract (1000 μg/mL) and the bacterial suspension (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*), were incubated during 12 h at 37±2 ºC. Gentamicin (16 μg/ mL) was employed as positive control. The OD_{620} was determined after the incubation period and bacterial proliferation was estimated according to the maximum growth control. The IC_{50} was determined when the extract it inhibited over 50% of bacterial growth. The *C. alba* extract was dissolved in dimethyl sulfoxide.

Statistical analysis

Results were expressed as mean ± standard error of the mean (S.E.M) and analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's post hoc test, to determine the differences between groups. Values of $p < 0.05$ were considered significant. IC_{50} value was calculated using nonlinear regression analysis.

RESULTS AND DISCUSSION

Cordia is a genus of trees and bushes widely distributed in warm regions. Around 300 species have been identified all over the world. The plants of this genus are used in the traditional medicine as scaring, astringent, anthelmintic, antimalarial, diuretic, antibacterial and anti-inflammatory. Some species in the genus, like *Cordia cylindrostachya* Roem. & schult, *Cordia myxa* L., and *Cordia verbenacea* DC, possess scientifically proven traditional uses as anti-inflammatory and antibacterial agents (Thirupathi et al. 2008; Matias et al. 2015).

However, for the flowers of *Cordia alba*, this would constitute the first scientific report supporting the traditional use for these biological activities.

The TPA application produced a strong inflammatory response in the mice ear, with massive edema formation and influx neutrophils, which were significantly decreased by extract of the *Cordia alba* flowers, with inhibition values similar to those shown by the positive control, indomethacin (Table 4.1 and Figure 4.1).

Table 4.1: Effect of the total extract from *Cordia alba* flowers on TPA-induced ear edema model

Groups	Dose (mg/ear)	Ear weight increase (mg)	Inhibition %
Control (TPA + acetone)	–	13 ± 0.5	–
Indomethacin	0.5	5.8 ± 0.7 ***	55 ± 5.8
Cordia alba	1.0	7.5 ± 0.4***	42 ± 2.8

Results are expressed as mean ± SEM. (n = 6)
***$p < 0.001$ ANOVA, statistically significant vs control group (TPA + acetone)

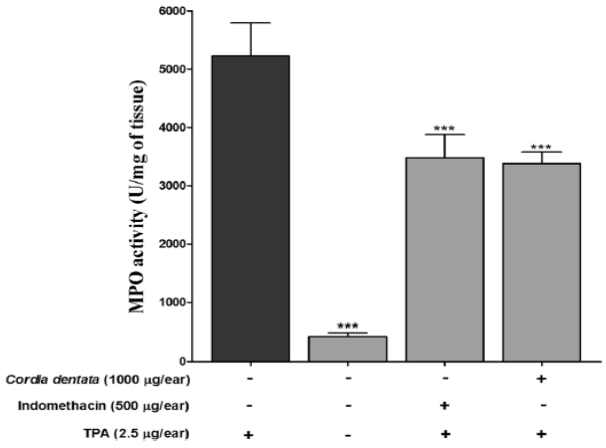

Figure 4.1: Effect of the total extract from *Cordia alba* flowers on MPO activity in ear tissue. Results are expressed as mean ± SEM. (n = 6) (***p < 0.001 ANOVA, statistically significant vs TPA group)

These results show the potential anti-inflammatory activity of this species. Neutrophils are cells that participate in the immune defense of the host against pathogens, they have been considered as the first line of defense for the organism, although they are also involved in the adaptive immunity. These cells constantly circulate the blood stream, until recruitment to the peripheral tissues in case of pathogen invasion or tissue damage, like the one caused by the TPA in the ear (Sadik et al. 2011; M.csai 2013). During the inflammatory response, neutrophils release enzymes like myeloperoxidase (MPO) and free radicals like ROS which contribute to host defense. MPO is one of the most abundant proteins in neutrophils; it is stored in the azurophil granules and is released after the activation of cells (Lee et al. 2003;

Metzler et al. 2011; Prokopowicz et al. 2012). Topical application of TPA activates protein kinase C, promoting the synthesis of chemokines by epidermal cells and therefore neutrophil infiltration (Nakadate 1989). *Cordia alba* is presented as a species with potential value in the treatment of chronic inflammatory diseases, since the total extract obtained from *Cordia alba* flowers reduced the neutrophil infiltration caused by the TPA, decreasing inflammation and minimizing the damage caused by the ROS produced by neutrophils in the tissue. Additionally, the extract may also do it directly by scavenging of ROS, as it showed a scavenging activity of the tested radicals in a concentration dependent manner, with IC_{50} values of 673.6 ± 1.5 μg/mL for DPPH and 276.7 ± 1.3 μg/mL for ABTS (Fig. 4.2).

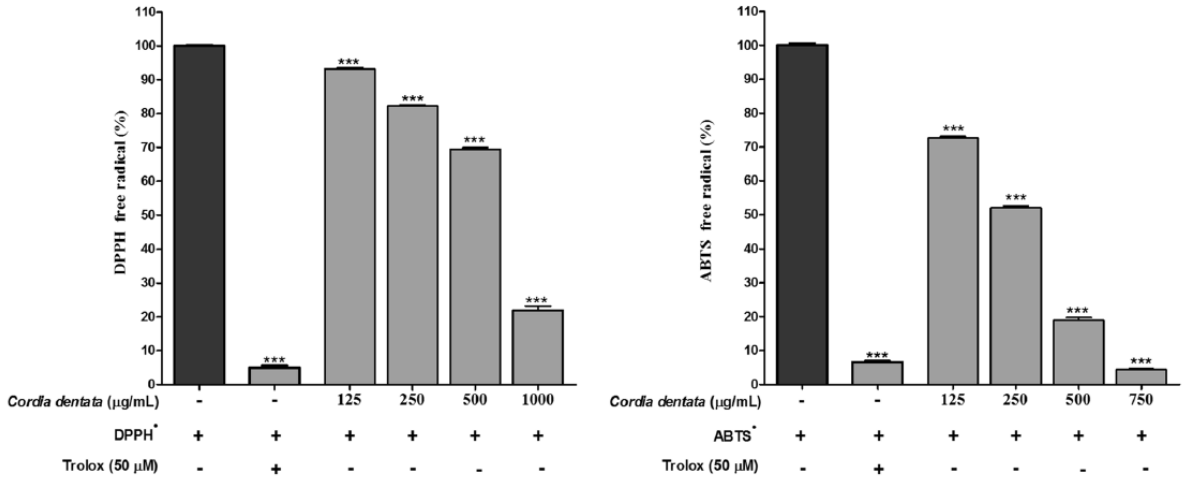

Figure 4.2: Scavenging effect of DPPH and ABTS free radicals by the total extract from *Cordia alba* flowers. Results are expressed as mean ± SEM. (n = 8) (***p < 0.001 ANOVA, statistically significant vs control group)

P. aeruginosa, *K. pneumoniae* and *S. aureus* affect mostly immunocompromised hosts, being responsible a high percentage of the development of nosocomial infections. Antibiotic resistance in these strains is now a really important issue that restricts the therapeutic alternatives (Shon et al. 2013; Sedighi et al. 2014). In this study, we demonstrated that the *Cordia alba* flowers extract has an inhibitory effect on the growth of *P. aeruginosa* and *S. aureus,* similar to other *Cordia species* (Table 4.2) (Okusa et al. 2007; Moura-Costa et al. 2012) and in accordance with reports from Cervantes et al. (2017), who evaluated different polarity extracts from the leaves of *C. alba*, finding that the extracts were able to inhibit the growth of *S. aureus*, *S. epidermidis*, *K pneumoniae* and *P. aeruginosa*. Similarly, Molina-Salinas et al. (2007) and Rodrigues et al. (2012) demonstrate the capacity of other species of the *Cordia* genus like *C. verbenacea* and *C. boissieri*, to inhibit the growth of *S. aureus*.

Strains	Inhibition %	IC_{50} (μg/mL)
P. aeruginosa	85.30 ± 3.51	107.04 (68.73–140.93)
S. aureus	39.71 ± 3.42	n.d
K. pneumoniae	9.14 ± 7.23	n.d
Gentamicin	74.16 ± 0.56	n.d

Results are expressed as mean ± SEM. (n = 8)

n.d. not determined

Table 4.2: Effect of the total extract from *Cordia alba* flowers against *P. aeruginosa*, *K. pneumoniae* and *S. aureus* growth

Metabolite	*Cordia alba* extract
Alkaloids	–
Coumarins	+++
Flavonoids	+++
Cardiotonic glycosides	++
Leucoanthocyanidins	–
Phenolic compounds	+++
Quinones	–
Saponins	–
Tannins	+++
Triterpenoid/steroids	+++

– not detected, ++ indicates moderate presence, +++ indicates high presence

Table 4.3: Phytochemical constituents of *Cordia alba* flowers

The anti-inflammatory and antibacterial activity showed by the extract of the flowers of *Cordia alba* might be related to the synergic action of flavonoids, triterpenoids, phenolic compounds, tannins and coumarins; metabolites reported by their anti-inflammatory, antioxidant and antibacterial properties, that have also been found in other species of the *Cordia genus* (Table 4.3) (Cowan 1999; Thirupathi et al. 2008; Kurek et al. 2011; Kumar and Pandey 2013; Venugopala et al. 2013).

The anti-inflammatory activity of the flowers of *Cordia alba* was demonstrated, as well as its capacity to inhibit the growth of *P. aeruginosa* and *S. aureus*, establishing the first scientific report that support the traditional use of this species for these biological activities.

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CAPÍTULO 2: Frutas consumidas en Colombia con efecto antioxidante y anti-inflamatorio

En este capítulo se presenta evidencia científica del potencial antioxidante y anti-inflamatorio de 10 extractos de frutas que crecen y se consumen en Colombia. El extracto del fruto de *Physalis peruviana* (Uchuva) resultó ser el más activo y se procedió a evaluar el efecto inmunodulador de una dieta suplementada con dicho extracto, en el modelo de enfermedad inflamatoria intestinal inducida por dextrán sulfato de sodio (DSS) en ratones BALB/c. Estos resultados se encuentran consignados en los productos 5 y 6 de esta tesis doctoral, los cuales se describen a lo largo de este capítulo.

A continuación, se presentan los productos de generación de nuevo conocimiento relacionados con esta evaluación:

Producto V: Mejía, N. M., Castro, J. P., Ocampo, Y. C., Salas, R. D., Delporte, C. L., & Franco, L. A. (2020). Evaluation of antioxidant potential and total phenolic content of exotic fruits grown in Colombia. *Journal of Applied Pharmaceutical Science*, *10*(09), 050-058.

Producto VI: Castro, J., Cortez, A., Lopez, G., Aguilera, J., De la Puerta, R., Salas, R., Barrios, L., Franco, L. Golden berry fruit modulate inflammation on (DSS)-induced acute colitis and RAW 264.7 macrophages. Artículo en Preparación.

PRODUCTO V:

EVALUATION OF ANTIOXIDANT POTENTIAL AND TOTAL PHENOLIC CONTENT OF EXOTIC FRUITS GROWN IN COLOMBIA

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ABSTRACT

Disruption of the balance between reactive oxygen species (ROS) production and endogenous antioxidant defenses leads to oxidative stress, which is related to the appearance of chronic noncommunicable diseases (NCDs). The antioxidant compounds present in fruits, including phenolic compounds, suggest that high fruit consumption may contribute to counteracting oxidative stress. The antioxidant potential of ten fruits grown in Colombia was evaluated by quantifying the content of total phenolic compounds, as well as their free radical scavenging capacity [2,2-diphenyl- 1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), peroxyl radicals (ROO), and nitric oxide (NO)], the reduction capacity of ferric reducing antioxidant power (FRAP), and the inhibition of the generation of ROS using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method in RAW 264.7 mouse macrophages. Regarding phenolic compounds, the extracts of banana passion and sour guava presented the highest content. These extracts also presented the most potent ferric-reducing ability and the best scavenging activity of DPPH, ABTS, NO, and ROO. Moreover, banana passion, yellow pitaya, and golden berry extracts significantly inhibited the production of ROS on LPS-stimulated macrophages. Our results show that banana passion, yellow pitaya, and golden berry are essential sources of molecules with antioxidant activity and their intake could modulate oxidative stress and consequently could help prevent or reduce the incidence of NCDs.

Key words: Fruits, phenolic compounds, oxidative stress, antioxidant activity.

INTRODUCTION

The generation of reactive oxygen species (ROS) by the body's metabolism has a beneficial role in killing infectious agents and regulating cell signaling pathways, as well as genes related to the inflammatory and immune response (Khurana et al., 2013). Nevertheless, the imbalance produced by this intracellular accumulation of ROS induces oxidative stress, which is a cellular state in which DNA, carbohydrates, proteins, and lipids are oxidized,

resulting in impaired cellular function, mutations, and cell death. These cellular alterations have been related to the initiation and progression of diseases such as diabetes, cancer, cardiovascular disease, and Alzheimer's disease, among others that are classified as noncommunicable diseases (NCDs) (Lee et al., 2015; Liguori et al., 2018). In this sense, substances capable of inhibiting the excessive production of ROS could become an alternative to reduce the incidence of NCDs.

Results obtained in epidemiological studies showed a correlation between the high consumption of fruits and the prevention of NCDs, especially cardiovascular diseases and cancer (Aune et al., 2017; Boeing et al., 2012; Wang et al., 2014). Based on these studies, the World Health Organization has recommended the consumption of five servings of fruits and vegetables daily as per the Strategy for the Prevention and Control of NCDs (Aune et al., 2017). Consistent with this recommendation, the relationship between fruit consumption and health promotion has been evidenced by the increase in the number of bibliographic references. These studies concluded that the positive effect of fruits on health is associated with their content of phytochemicals, mainly phenolic compounds (Côté et al., 2010; Hidalgo and Almajano, 2017; Kalaycıoğlu and Erim, 2017; Skrovankova et al., 2015). These compounds have a recognized antioxidant effect and, therefore, have been associated with the prevention of NCDs (Y Aboul-Enein et al., 2013; Zhang et al., 2015).

The demand for fruits in national and international markets has continuously grown, perhaps by the publications that show that low consumption of fruits is among the main factors associated with worldwide mortality. On the contrary, fruit intake is related to a decreased risk of NCDs (Khoo et al., 2011; Slavin and Lloyd, 2012). Colombia is one of the countries that is considered as megadiverse, since it has a great variety of exotic fruits (Contreras-Calderón et al., 2011). Fruits such as yellow pitaya (*Hylocereus megalanthus*), banana passion (*Passiflora cumbalensis*), purple passion fruit (*Passiflora edulis*), golden berry (*Physalis peruviana*), tamarillo (*Solanum betaceum*), lulo (*Solanum quitoense*), soursop (*Annona muricata*), kalipatti sapota (*Manilkara zapota*), sapote (*Pouteria sapota*), and sour guava (*Psidium friedrichsthalianum*) are commonly consumed in Colombia and are used extensively to treat various diseases in different regions of the world. For example, soursop and purple passion fruit are used to treat cancer, hypertension, inflammation, among other diseases (Daddiouaissa and Amid, 2018; Nanda et al., 2013; Taïwe and Kuete, 2017). Sapote and tamarillo are useful remedies for patients' gastrointestinal problems (Angulo et al., 2012; Ngomle et al., 2020; Stanley et al., 2009). Golden berry is used to treat asthma, rheumatism, cancer, and leukemia (Hassanien, 2011; Wu et al., 2004a). Lulo is used to treat nerve problems and skin diseases (Bussmann et al., 2018). Kalipatti sapota is used in the treatment of pulmonary diseases and also inhibits the growth of breast and colon cancers (Bano and Ahmed, 2017). Yellow pitaya has a mild laxative effect (Fratoni et al., 2019; Sudha et al., 2017). Leaves and flowers of *P. cumbalensis* are widely employed in folk medicine as a

poultice for gastrointestinal problems (Jerves-Andrade et al., 2014). However, no reports of the species of *P. friedrichsthalianum* were found in the literature.

Regarding studies of antioxidant activity, in the literature, there are reports of the antioxidant potential of some fruits included in this study that are evaluated individually. For example, the content of total phenolic compounds and the uptake of radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) have already been reported for all fruits included in this work (Bravo et al., 2016; dos Reis et al., 2018; Flores et al., 2013; Gordillo et al., 2012; Prabhu et al., 2018; Torres Grisales et al., 2017; Vasco et al., 2008; Woo et al., 2013), while for yellow pitaya and sour guava, there are no activity reports for the ferric-reducing antioxidant power (FRAP) or the oxygen radical absorbance capacity-fluorescein (ORAC-FL) (Bravo et al., 2015, 2016; Espin et al., 2016; González et al., 2012; Gordillo et al., 2012; Penaloza et al., 2017; Shafii et al., 2017; Vasco et al., 2008; Woo et al., 2013; Yahia and Barrera, 2009; Yahia et al., 2011; Zapata et al., 2014). Moreover, the scavenging of nitric oxide radical and the inhibition of the generation of intracellular ROS have been reported only for golden berry, kalipatti sapota, soursop, tamarillo, and purple passion fruit (Gomathy et al., 2013; González et al., 2016; Kou et al., 2009; Maruki-Uchida et al., 2013; Orqueda et al., 2020; Rop et al., 2012; Tan et al., 2018; Wu et al., 2004b; Zamudio-Cuevas et al., 2014). Despite this background, it is complicated to determine which would be the most promising fruits as antioxidants, since the fundamental differences are observed in the extraction methods regarding the used methodology and in the expression of the results. Few reports have studied the antioxidant properties of these fruits under the same experimental conditions (Bravo et al., 2016; Contreras-Calderón et al., 2011). Taking into account the high demand for these fruits, their ethnopharmacological use, and antioxidant history, in this work, we evaluated the antioxidant potential of ten exotic fruits grown in Colombia to identify the promising fruits for the treatment or prevention of NCDs by quantifying the content of total phenolic compounds, as well as their free radical scavenging capacity (DPPH, ABTS, ROO, NO). Additionally, the reduction capacity of the extracts was measured with the FRAP assay and the inhibition of the generation of ROS in lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophages was measured using the DCFH-DA method.

MATERIALS AND METHODS

Reagents

Gallic acid, Folin–Ciocalteu, sodium nitroprusside dihydrate, sulfanilamide, horseradish peroxidase, guaiacol, catechin, fluorescein, quercetin, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ABTS, DPPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ),

5,5-dimethyl-1-pyrroline N-oxide, N-(1-naphthyl)ethylenediamine dihydrochloride, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), pyrogallol red, ferric chloride, ferrous sulfate heptahydrate, magnesium sulfate heptahydrate, potassium persulfate, and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO). Phosphoric acid, potassium chloride, and sodium chloride were obtained from JT Baker (Phillipsburg, NJ). Methanol, ethanol, dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Caffeic acid, aluminum chloride, ascorbic acid, sodium hydroxide, sodium nitrite, D-glucose, calcium chloride, potassium phosphate monobasic, potassium phosphate dibasic, sodium phosphate dibasic, sodium phosphate monobasic, hydrochloric acid, acetic acid, hydrogen peroxide, and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Merck Millipore (Burlington, MA).

Fruit samples and determination of physicochemical parameters

Fruits included in this study were obtained from Fusagasugá (Cundinamarca) and Turbana (Bolívar), which involved yellow pitaya (*H. megalanthus*), banana passion (*P. cumbalensis*), purple passion fruit (*P. edulis*), golden berry (*P. peruviana*), tamarillo (*S. betaceum*), lulo (*S. quitoense*), soursop (*A. muricata*), kalipatti sapota (*M. zapota*), sapote (*P. sapota*) and sour guava (*P. friedrichsthalianum*). Representative samples of each fruit were used to determine the soluble solids (ºBrix), titratable acidity, and pH values, which were conducted following the guidelines established by the Association of Official Analytical Chemists (Helrich, 1990). The concentration of the solid soluble content (ºBrix) was measured using a digital refractometer (Sper Scientific 300034) at 25°C and the pH values were determined by a pH meter (Starter 3100, OHAUS) at 25°C.

Preparation of extracts and determination of total phenolics content

Fruits were washed under tap water, weighed, homogenized, frozen at −80°C, and lyophilized. The dried material was extracted with ethanol (96%) at room temperature in stoppered containers for a defined period of time with frequent agitation until exhaustion and the obtained extract was concentrated in a rotary evaporator at controlled temperatures (38°C – 40°C) and reduced pressure. The Folin–Ciocalteu method was used to quantify the total phenolic compounds (Rivera et al., 2018). Phenolic compounds were calculated as mg gallic acid equivalents (GAE)/100 g of fresh weight.

Antioxidant activity assays

DPPH and ABTS free radical assays

Free radical DPPH and ABTS scavenging was determined using the standard methods with some modifications (Castro *et al*., 2018). For the uptake of the DPPH radical, 75 µl of different concentrations of the fruit extracts were mixed with 150 µl of a methanolic solution of DPPH (70 µg/ml). The mixture was allowed to incubate for 30 minutes and the absorbance at 517 nm was measured. While for the ABTS radical, 20 µl of the different concentrations of fruit extracts were mixed with 180 µl of the ABTS solution. After 30 minutes of incubation, the absorbance was measured at 734 nm in a Multiskan Go microplate reader (Thermo Scientific, Waltham, MA). A Trolox standard curve was used to determine the equivalent antioxidant capacity and the results were expressed as µmol Trolox equivalents/g of fresh weight.

Ferric-reducing antioxidant power assay

In this work, we used the standard method with some modifications (Castro et al., 2014). The FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6) with 1 volume of 10 mM TPTZ in HCl (40 mM) and with 1 volume of 20 mM $FeCl_3 \cdot 6H_2O$. Different concentrations of fruit extracts (10 µl) were mixed with the FRAP reagent (290 µl), incubated independently at 37°C, and the absorbance was determined at 593nm after 60 minutes of the reaction using an EnSpire Multimode microplate reader (PerkinElmer, Waltham, MA). The FRAP value was determined by plotting a standard curve made by the addition of ferrous sulfate to the FRAP reagent. Results were expressed as µmol of Fe^{+2}/100 g of fresh weight.

Oxygen radical absorbance capacity-fluorescein

The ability of fruit extracts to trap the peroxyl radical was determined according to the method proposed by Ou et al. (2001) and validate by Bisby et al. (2008). The peroxyl radicals generated by the thermal decomposition of AAPH reacted with fluorescein to produce a nonfluorescent product over time. Briefly, 20 µl of different concentrations of fruit extracts were preincubated for 5 minutes with 120 µl of fluorescein (100 nM) in a 96-well flat-bottom black plate. After which, 60 µl of AAPH (600 mM) was added to each well and the fluorescence was recorded using a Fluoroskan Ascent equipment (Thermo Scientific, Waltham, MA) every 56 seconds for 150 minutes at excitation and emission wavelengths of 485 and 538 nm, respectively. The results were expressed as µmol Trolox equivalents/g of fresh weight.

Hydrogen peroxide scavenging activity

The method described by Doerge et al. (1997), with some modifications, was used to determine the hydrogen peroxide scavenging activity of fruit extracts. Briefly, 900 µl of HBSS buffer, 100 µl of H_2O_2 (10 mM), and 10 µl of fruit extracts at different concentrations were incubated for 30 minutes. After incubation, 50 µl of guaiacol and 10 µl of horseradish peroxidase (0.6 µg/ml) were added and incubated again for 20 minutes. The scavenging activity was determined in a Multiskan EX microplate reader (Thermo Scientific, Waltham, MA) at 450 nm. Results were expressed as µmol Trolox equivalents/g of fresh weight.

Scavenging of the nitric oxide radical

Nitric oxide is generated by solubilizing sodium nitroprusside in an aqueous solution, where it is transformed into nitrite by the oxygen present in the medium. The produced nitrites are easily quantified by the spectrophotometric assay based on the Griess reaction (Rao, 1997). We incubated 990 µl of sodium nitroprusside solution (10 mM) in PBS with 10 µl of different concentrations of fruit extracts for 4 hours at room temperature. After incubation, equal volumes of Griess reagent and the samples were mixed. The nitric oxide concentration was determined by spectrophotometry at 550 nm in a Multiskan Go microplate reader (Thermo Scientific, Waltham, MA) by comparing to a standard curve of $NaNO_2$ (1–200 µM). Results were expressed as µmol of results were expressed as µmol of caffeic acid equivalents/g of fresh weight.

Cellular ROS determination by DCFH-DA

Cell culture

Murine macrophage RAW cell lines (ATCC® TIB-71™, Rockville, MD) were maintained in the DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere at 37°C.

Assessment of cell viability

The toxicity of fruit extracts on RAW 264.7 macrophages was evaluated using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric method (Scudiero et al., 1988). RAW 264.7 macrophages were seeded in sterile 96-well plates (2×10^4 cells/well) and incubated at 37°C for 48 hours, then the medium was removed, and cells were washed with phosphate buffer saline (PBS) and treated for 30 minutes with different concentrations of fruit extracts, after which, they were activated with LPS (1 µg/ml) and incubated again for 24 hours at 37°C. Following exposure, the cell culture medium was discarded and 100 µl of MTT solution (0.25 mg/ml) was added to each well of the plate. The

plates were incubated at 37ºC for 4 hours in the CO_2 incubator. Finally, 100 µl of DMSO was added to dissolve the formazan crystals, and the absorbance was measured at 550 nm by a Multiskan EX microplate reader (Thermo Scientific, Waltham, MA). In each trial, a group of cells not exposed to the extracts was included as the negative control and a group exposed to Triton X-100 (20%) as control of maximum toxicity. The viability percentages were calculated, considering the negative control group as 100% viability**.**

DCFH assay

The formation of intracellular ROS was determined using the oxidation-sensitive dye DCFH-DA according to the protocol described by Park *et al.* (2017), with some modifications. For that, macrophages RAW 264.7 (10×10^4 cells/well) were incubated in 96-well sterile black plates. After 24 hours of incubation, the cells were washed twice with sterile PBS to remove nonadherent cells. The remaining cells were incubated for 30 minutes with different concentrations of fruit extracts and activated with LPS (1 µg/ml). After 24 hours of incubation, the cells were washed three times with sterile PBS, stained with DCFH-DA (20 µM) in PBS, and incubated for 30 minutes at 37°C in the dark. Fluorescence intensity was measured in a 96-well Fluoroskan Ascent plate reader (Thermo Scientific, Waltham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Quercetin was used as the positive control.

Statistical analysis

Antioxidant activity results of three independent assays were reported as mean ± SEM. A one-way analysis of variance was used to analyze the data, followed by Tukey's and Dunnett's comparison tests. *P*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Fruits, like vegetables, provide nutrients, such as vitamins and minerals, as well as nonnutritive secondary metabolites with biological activity that can contribute to improving people's health and reducing the risk of chronic NCD. Among these metabolites, phenolic compounds stand out, which have several pharmacological properties and are recognized for their potent antioxidant activity (Liu, 2013). In this work, we evaluated the antioxidant potential as well as the content of total phenolic compounds of ten extracts of fruits grown in Colombia using seven different methods. The information of fruits selected for this study and their state of maturation is presented in Tables 5.1 and 5.2.

Table 5.1: Information on fruits included in this study

Fruits	Part used	Extract yields (%)	Voucher number	Collection site
Yellow pitaya *Hylocereus megalanthus*	Pulp and seed	24.54	199083	Fusagasugá (Cundinamarca) (4°20′14″ N, 74°21′52″ O) Altitude: 1,800 masl Precipitation: 950 mm of rain per year Climate: 8°C–28°C
Banana passion *P. cumbalensis*	Pulp	9.78	199085	
Purple passion fruit *P. edulis*	Pulp	13.04	199081	
Golden berry *P. peruviana*	Whole fruit	18.08	199086	
Tamarillo *S. betaceum*	Pulp	9.10	199080	
Lulo *S. quitoense*	Pulp and seed	5.46	199084	
Soursop *A. muricata*	Pulp	13.12	201951	Turbana (Bolívar) (10°16′35″ N, 75°26′19″ O) Altitude: 138 masl Precipitation: 778 mm of rain per year Climate: 23°C–32°C
Kalipatti sapota *M. zapota*	Pulp	32.27	201955	
Sapote *P. sapota*	Pulp	26.26	201953	
Sour guava *P. friedrichsthalianum*	Pulp	12.01	201952	

The part of the fruit used to carry out the study and prepare the extracts was selected based on the way people eat the fruit. Yields were calculated as: [(grams of lyophilized pulp/grams of fresh weight) × 100].

Table 5.2: Physicochemical parameters of the fruits.

Fruits	Brix	pH	Total acidity (g/100 ml)
Yellow pitaya	17.68 ± 0.27	4.58 ± 0.01	0.11 ± 0.01
Banana passion	10.62 ± 0.11	3.41 ± 0.03	1.23 ± 0.04
Purple passion	15.04 ± 0.46	3.13 ± 0.04	2.97 ± 0.25
Golden berry	14.20 ± 0.06	3.52 ± 0.006	2.15 ± 0.01
Tamarillo	9.61 ± 0.14	3.61 ± 0.004	2.18 ± 0.03
Lulo	9.08 ± 0.16	3.13 ± 0.007	3.33 ± 0.09
Soursop	12.57 ± 0.38	4.06 ± 0.04	0.62 ± 0.04
Kalipatti sapota	25.37 ± 0.30	5.25 ± 0.08	1.80 ± 0.18
Sapote	22.33 ± 0.37	6.75 ± 0.05	1.07 ± 0.02
Sour Guava	8.43 ± 0.20	2.86 ± 0.02	3.80 ± 0.09

The results represent mean ± SEM ($n = 9$) from three independent experiments.

Quantification of phenolic compounds

Results of the quantification of phenolic compounds of fruits under study are presented in Table 5.3. Considering the broad range of variations in the total phenolic compounds, the fruits were classified into three groups: with a high content (>80 mg GAE/100 g FW), a moderate content (80> mg GAE/100 g FW >40), and a low content (<40 mg GAE/100 g FW). The results show that only banana passion and sour guava presented high contents of phenolic compounds. On the contrary, yellow pitaya, golden berry, purple passion fruit, sapote, kalipatti sapota, tamarillo, and soursop showed moderate content. Lulo had the lowest content of these compounds.

Table 5.3: Quantification of phenolic compounds and antioxidant potential of the extracts by the DPPH, ABTS, and FRAP methods.

Fruits	Phenolic compounds	DPPH	ABTS	FRAP
Banana passion	249.5 ± 0.064	1959.7 ± 1.288	4257.3 ± 0.622	5962.6 ± 3.241
Sour guava	80.9 ± 0.010	374.5 ± 0.083	822.3 ± 0.110	1607.4 ± 0.220
Yellow pitaya	59.1 ± 0.013	177.1 ± 0.073	323.8 ± 0.026	811.2 ± 0.201
Golden berry	53.3 ± 0.011	78.9 ± 0.018	228.6 ± 0.034	419.7 ± 0.125
Purple passion fruit	50.3 ± 0.006	93.3 ± 0.030	209.8 ± 0.027	312.4 ± 0.149
Sapote	49.0 ± 0.004	136.5 ± 0.030	310.7 ± 0.015	549.2 ± 0.094
Kalipatti sapota	44.7 ± 0.008	73.2 ± 0.034	193.9 ± 0.044	412.2 ± 0.156
Tamarillo	41.9 ± 0.007	72.0 ± 0.018	197.6 ± 0.023	427.2 ± 0.070
Soursop	40.4 ± 0.008	64.3 ± 0.018	183.8 ± 0.023	204.1 ± 0.091
Lulo	31.8 ± 0.006	84.5 ± 0.036	144.2 ± 0.008	341.2 ± 0.147

Phenolic compounds were calculated as mg gallic acid/100 g of fresh weight. Results of DPPH and ABTS were expressed as micromoles of Trolox equivalents (TEs) per 100 g of fresh weight (µmol of TEs/100 g of FW) and FRAP results as micromoles of Fe^{+2} per 100 g of fresh weight (µmol of Fe^{+2}/100 g of FW). Results represent mean ± SEM (n = 12) from three independent experiments.

DPPH, ABTS, and FRAP assay

Table 5.3 presents the results of the scavenger capacity of DPPH and ABTS free radicals as well as the FRAP of the ten fruit extracts under study. It can be observed that the extracts of banana passion and sour guava presented the best scavenging activity of radicals DPPH and ABTS and the best activity of ferric ion reduction. On the contrary, the extracts of yellow pitaya, golden berry, purple passion fruit, sapote, kalipatti Sapota, tamarillo, soursop, and lulo had a moderate activity. These results are related to the content of phenolic compounds, showing high correlation coefficients in all cases (DPPH: r = 0.978; ABTS: r = 0.979; and FRAP: r = 0.981). These results are similar to those reported by other studies, where the free radical scavenging capacity of phenolic compounds is evident; being able to react with highly

ROS, donating a hydrogen atom (HA), or transferring a single electron (SE) is all due to the low redox potential that it possesses (Kurek-Górecka *et al*., 2014). In fact, according to reaction mechanisms, antioxidant activity tests can be classified as a transference assay of an HA or an SE. FRAP and ABTS have been included in the category as the HA assays, while the DPPH assay can be classified in both groups since both reactions can occur, and the antioxidant agent determines the final mechanism (Hidalgo and Almajano, 2017). It should be noted that although the extracts of golden berry, purple passion fruit, and sapote showed a similar content of phenolic compounds, sapote extract presented better free radical scavenging capacity. This may happen because the activity of phenolic compounds also depends on the concentration on the number of hydroxyl groups and the position of the aromatic ring (Kurek-Górecka *et al*., 2014).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) scavenging assays

The excessive production of ROS causes cell toxicity and target organ damage, causing the initiation of NCDs. The results of ROS scavenging activity by those fruit extracts showed that the extracts of yellow pitaya, sour guava, and banana passion exhibited potent peroxyl radical scavenging activity (Table 5.4). Extracts of sour guava and banana passion significantly decrease the amount of H_2O_2, which is an important precursor of hydroxyl radicals among other free radicals.

The NO radical can act directly as an effector molecule or as a second messenger, intervening in numerous signaling pathways (Zhang *et al*., 2017). When it is produced in excess, it can cause noxious effects, which are related to the capacity of this mediator to react with oxygen, superoxide, and transition metals, generating NOx, peroxynitrite, and heme-complexes, respectively. Peroxynitrite is a powerful, extremely cytotoxic oxidizing agent used by cells of the immune system within its defense mechanisms. However, when produced in excess, it can damage a wide range of molecules in cells, including proteins, DNA, and lipids, which leads to the initiation of a recognized pathophysiological mechanism causing the appearance of chronic NCDs (Pacher *et al*., 2007). Extracts of sour guava and banana passion presented an important nitric oxide radical scavenging effect (Table 5.4). It is remarkable that, once again, the fruit extracts with the major activity were those with the highest content of phenolic compounds.

Production of intracellular ROS

The DPPH, ABTS, FRAP, and ORAC-FL assays are widely used to evaluate the antioxidant capacity of fruits (Hidalgo and Almajano, 2017). However, in recent years, antioxidant activity assays that include cellular models have been widely accepted since it is considered that the results obtained with them could better predict the antioxidant power of fruits.

Table 5.4: Scavenging effect on reactive oxygen and nitrogen species

Fruits	ORAC	H_2O_2	NO
Banana passion	2409.82 ± 120.74	38.04 ± 1.71	70.81 ± 8.58
Sour guava	2602.08 ± 51.94	36.51 ± 1.90	168.81 ± 3.60
Yellow pitaya	2999.77 ±80.56	2.38 ± 0.01	ulq
Golden berry	350.13 ± 5.54	4.78 ± 0.44	ulq
Purple passion fruit	471.69 ± 4.91	13.79 ± 3.80	ulq
Sapote	143.87 ± 2.65	2.63 ± 0.15	31.50 ± 4.22
Kalipatti sapota	697.69 ± 7.82	0.66 ± 0.14	ulq
Tamarillo	169.71 ± 3.15	4.94 ± 0.21	18.41 ± 3.75
Soursop	311.24 ± 3.69	3.84 ± 0.01	ulq
Lulo	133.19 ± 1.84	1.99 ± 0.06	37.81 ± 1.44

ORAC and H_2O_2 scavenging capacities were expressed as micromoles of Trolox equivalents per gram of fresh weight (µmol of TEs/g of FW). NO scavenging capacity was expressed as micromoles of caffeic acid equivalents per gram of fresh weight (µmol of caffeic acid/g of FW). Results represent mean ± SEM (n = 12) from three independent experiments. Ulq = Under limit of quantification.

In order to deepen the understanding of the antioxidant potential of the ten fruit extracts, their capacity to inhibit the generation of intracellular ROS was measured using DCFH-DA. The extracts of golden berry, banana passion, and yellow pitaya were the most active (Figure 5. 1). The extracts of sour guava, purple passion fruit, sapote, kalipatti sapota, tamarillo, and lulo showed a low inhibition on the formation of intracellular ROS (data not shown). The extract of soursop fruit was not evaluated because it had a toxic effect on the cells. Note that the extract of golden berry was more active than expected, as it was not the extract with the highest content of phenolic and it did not have the best free radical scavenging effect. The opposite happened with the extract of sour guava, which showed the best free radical scavenging activity and the highest content of phenolic compounds, but it did not exhibit a high capacity to inhibit the production of ROS in the cell assay. In such a way that there is no evidence of a correlation between antioxidant activity at the cellular level and the content of phenolic compounds, contrary to what happens with DPPH, ABTS, and FRAP free radical scavenging assays. It could be because the antioxidant properties of polyphenols depend on the physicochemical environment, which is very complex within a biological matrix, while it is more stable in ABTS, DPPH, and FRAP free radical scavenging assays (Scalbert *et al.*, 2005). Furthermore, it is also possible that the antioxidant activity shown by these fruit extracts is due to the presence of other secondary metabolites.

This study provides new information on the antioxidant properties of yellow pitaya, banana passion, lulo, sapote, and sour guava. The results of the scavenging effect on DPPH, ABTS, NO, and peroxyl radicals, as well as the inhibition of the generation of intracellular ROS in macrophages, showed that the total ethanolic extracts obtained from the edible parts of banana passion (*P. cumbalensis*), yellow pitaya (*H. megalanthus*), and golden berry (*P. peruviana*) make them the most promising fruits as antioxidants. These results support the

ethnopharmacological use of these three fruits, concerning the treatment of various chronic NCDs, in addition to being an essential foundation to continue promoting their consumption.

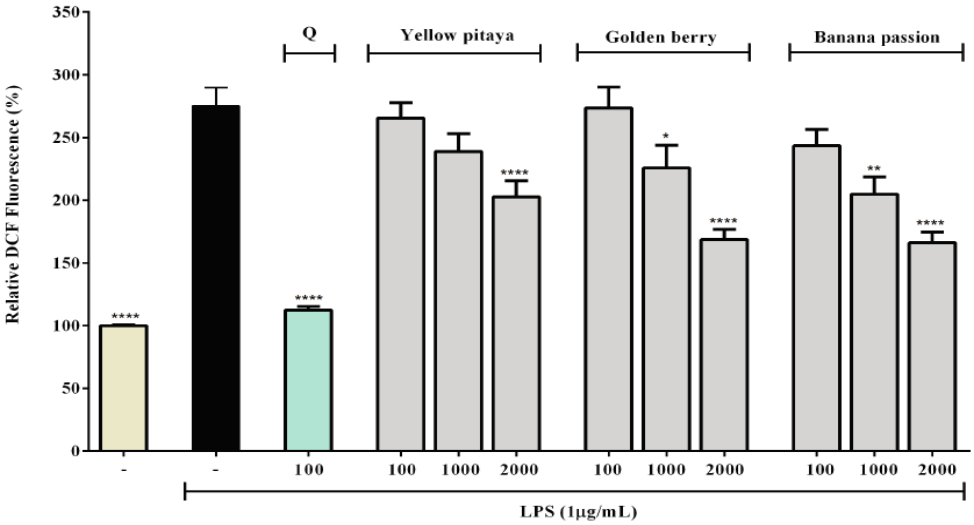

Figure 5.1: Effect of fruit extracts on the production of intracellular ROS in LPS-stimulated RAW 264.7 macrophages (1 µg/ml) for 24 hours. ROS are expressed as fold increase of 2′,7′-dichlorofluorescein fluorescence over LPS-untreated controls. Fruit extracts were evaluated to 100, 1,000, and 2,000 µg/ml. Quercetin (Q) (100 µM) was employed as the positive control. The results represent mean ± SEM. (*$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ are statistically significant compared to the LPS-treated group).

CONCLUSION

The fruits like banana passion (*P. cumbalensis*), yellow pitaya (*H. megalanthus*), and golden berry (*P. peruviana*) are a significant source of antioxidant compounds. With the results obtained in this work, we can propose these fruits as functional foods that can be used to enhance health. Nevertheless, these fruit extracts should be evaluated in *in vivo* models to ensure the bioavailability of the active compounds and should be studied more in-depth regarding their phytochemical characterization to identify the compounds responsible for this activity.

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PRODUCTO VI:

GOLDEN BERRY FRUIT MODULATE INFLAMMATION ON (DSS)-INDUCED ACUTE COLITIS AND RAW 264.7 MACROPHAGES

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ABSTRACT

Chronic non-communicable diseases (NCD) like diabetes, inflammatory bowel disease, cancer, among others, are the main cause of morbidity and mortality of the world population. NCD incidence is linked to modifiable risk factors, such as diet. Since the diet components can modify the composition of the microbiota and the immune response, playing in this way a fundamental role in intestinal homeostasis. The high consumption of fruit has been associated with decreasing the risk of developing NCD. Physalis peruviana L. is native to South American and is one of the best-known species of this genus. It is widely used in folk medicine for the treatment of inflammatory diseases. In this work we evaluated the anti-inflammatory potential *in vitro* of ten ethanolic extracts obtained of fruits grown in Colombia, determining their ability to inhibitory of nitric oxide ·NO production on LPS-activated RAW 264.7 macrophages. Additionally, we determined the effects of the four most active extracts on the production of IL-1β, IL-6, TNF-α and PGE-2 in RAW 264.7 macrophages activated with LPS, since, we hypothesized that the fruit that inhibits the greatest number of inflammatory mediators could have an immunomodulatory effect when it was intake regularly in the diet. To test this hypothesis, we evaluated whether the intake during 30 days of the most active fruit (golden berry) may modulate the inflammatory response in acute colitis induced by sodium dextran sulfate (DSS) in BALB/c mice. Our results show that purple passion, yellow pitaya, tamarillo and golden berry extracts significantly inhibited the production of IL-1β, IL-6, TNF-α and PGE-2 in RAW 264.7 macrophages activated with LPS, being the extract of golden berry the most promising as an anti-inflammatory and therefore its immunomodulatory effect was evaluated in a model of inflammatory bowel disease. The dietary supplementation with gold berry extract attenuates the pathological symptoms of acute colitis induced by sodium dextran sulfate (DSS) in BALB/c mice. Golden berry extract reduces bleeding, weight loss, diarrhea and shortening of the colon. The mechanisms of intestinal anti-inflammatory effect of golden berry can be associated with the inhibition of MPO enzyme activity and the decrease in production IL-6, IL-1β, IL-10, TNF-α and ROS.

INTRODUCTION

The induction of an inflammatory response does not necessarily require overt tissue injury or infection, this can also be started by signals released from stressed or malfunctioning cells. This cell states can be triggered by various factors, among which are those related to lifestyle such as physical inactivity, harmful consumption of alcohol, smoking and unhealthy diet (Medzhitov, 2008; Ruiz-Núñez et al., 2013).

When tissues are in conditions of stress, or when they malfunction for other reasons, they might send a different set of signals to tissue-resident macrophages than those sent by tissues in the basal state, if the changes are considerable, then adaptation to the conditions requires the help of tissue-resident or recruited macrophages and might require small-scale delivery of additional leukocytes and plasma proteins, which lead to a chronic low-grade inflammatory state, that can be defined as adaptive response intermediate between basal and inflammatory states (Medzhitov, 2008). This type of response is known as para-inflammation and it is associated with the development of various chronic non-communicable diseases (NCDs) such as inflammatory bowel disease, cardiovascular disease, cancer, respiratory diseases, diabetes mellitus, and neurodegenerative (Pan et al., 2010). Although the association between inflammation and chronic conditions is widely recognised, the issue of causality and the degree to which inflammation contributes and serves as a risk factor for the development of disease remain unresolved (Minihane et al., 2015).

Non-communicable diseases are the main cause of mortality globally. It is estimated that 70% of all deaths worldwide are attributable to non-communicable diseases (Holt et al., 2018; Ruby et al., 2015). A few decades ago, these diseases were very popular in developed countries due to unhealthy diet and physical inactivity (Wagner & Brath, 2012). However, it has been observed a rapid increase of non-communicable diseases in low- income and middle- income countries and already has major adverse social, economic, and health effects (Alwan et al., 2010). Non-communicable diseases are non-infectious diseases and long-lasting (more than three or six months). Most they can only be treated but not cured. Generally, they develop slowly and asymptomatically but lead to devastating complications, resulting in premature death and poor quality of life (Meetoo, 2008; Senthilkumar & Kim, 2013).

As mentioned previously, suboptimal nutrition ranks highest among lifestyle risk factors for NCDs globally and has been identified as the most important preventable NCDs risk factor. In recent decades, as part of globalization, Western lifestyles have been adopted throughout the world, including unhealthy dietary patterns, this trend is known as “nutrition transition”, characterized by replacement of a traditional diet rich in fruit and vegetables by a diet rich in calories provided by animal fats and low in complex carbohydrates. This has increased the incidence of NCDs worldwide (Astrup et al., 2008; Beaglehole & Yach, 2003; Kimokoti & Millen, 2016).

Epidemiological studies have shown that a high intake of fruits along with other foods as vegetable, grains and nuts has been useful to modulate the inflammatory process, decreasing in this way the circulating concentrations of inflammatory markers as: MCP-1, PGE-2, CRP, TNF-α, IL-8, IFN-α and IL-6. In this sense a high intake of fruits can prevent the appearance of NCDs or beneficially affect the quality of life of those who suffer it (Calder et al., 2011, 2017). The anti-inflammatory activity that some fruits can have, it is due to bioactive substances present in these foods as phenolic compounds, saponins, alkaloids, polyunsaturated fatty acids, terpenoids, among others; these compounds can inhibit inflammation-related pathways (Adefegha, 2018; Calder et al., 2017; Prasad et al., 2012; Szostak et al., 2013; Zhu et al., 2018).

In this work, we evaluated the anti-inflammatory potential *in vitro* of ten ethanolic extracts obtained from fruits grown in Colombia, determining their ability to inhibit the nitric oxide ·NO production on LPS-activated RAW 264.7 macrophages. Additionally, we determined the effects of the four most active extracts on the production of IL-1β, IL-6, TNF-α, and PGE-2 in RAW 264.7 macrophages activated with LPS, since we hypothesized that the fruit that inhibits the greatest number of inflammatory mediators could have an immunomodulatory effect when it was included regularly in the diet. To test this hypothesis, we evaluated whether the intake during 30 days of the most active fruit (golden berry) may modulate the inflammatory response in acute colitis induced by sodium dextran sulfate (DSS) in BALB/c mice.

MATERIALS AND METHODS

Reagents

Penicillin-streptomycin, trypan blue, and lipopolysaccharide from Escherichia coli (LPS), sodium nitrite, N-[1,1-naphthyl] ethylenediamine dihydrochloride, sulfanilamide, TEMED, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), Phosphate Buffer Saline (PBS) tablets, O-Dianisidine, ethylenediaminetetraacetic acid (EDTA), Hydrogen peroxide (H_2O_2), indomethacin, dexamethasone, rofecoxib, hematoxylin and eosin, were purchased from Sigma Aldrich (St Louis, MO). Ethanol, dimethyl sulfoxide (DMSO), Dulbecco's modified eagle medium (DMEM), and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Acrylamide/bisacrylamide, Tris HCL, Bradford assay, precision plus protein standards dual color, Tris/Glycine/SDS Buffer, Tris/Glycine Buffer, Blotting-Grade Blocker were obtained from Bio-Rad. Bromide of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), hexadecyl-trimethylammonium bromide (HTAB), were obtained from Calbiochem® (San Diego, CA). Macrophages RAW 264.7 were acquired from the American Type Culture Collection (Manassas, VA). Phosphoric acid, was obtained from JT Baker (Phillipsburg, NJ). ELISA kits from eBiosciences. Western HRP substrate was obtained from Millipore and T-PER Tissue Protein Extraction Reagent was obtained from Thermo Scientific

Fruit samples and determination of physicochemical parameters

Fruits included in this study were obtained from Fusagasuga (Cundinamarca) and Turbana (Bolivar) and involved yellow pitaya (*Hylocereus megalanthus*), banana passion (*Passiflora cumbalensis*), purple passion fruit (*Passiflora edulis*), goldenberry (*Physalis peruviana*), tamarillo (*Solanum betaceum*), lulo (*Solanum quitoense*), soursop (*Annona muricata*), kalipatti sapota (*Manilkara zapota*), sapote (*Pouteria sapota*) and sour guava (*Psidium friedrichsthalianum*). The taxonomic identification was carried out in the Herbarium University of Antioquia, Medellin, Colombia; a voucher of each species was kept in that institution.

Preparation of extracts

Fruits were washed under tap water, weighed, homogenized, frozen at -80ºC and lyophilized. The dried material was extracted with ethanol (96%) at room temperature in stoppered containers for a defined period with frequent agitation until exhaustion and the obtained extract was concentrated in a rotary evaporator at controlled temperature (38-40ºC) and reduced pressure.

Fingerprint chromatograms of fruit extracts

HPLC analysis was performed with a Beckman Coulter HPLC instrument. Equipped with a pump and diode array detector (DAD). For the development of fingerprint chromatograms, an appropriate amount of each extract was weighed into a 1,5 mL centrifuge tube and solubilized in DMSO and vortexed for some minutes. The column used was a Gemini-NX C18 (150x4,6nm, 3 µm, Phenomenex). Column temperature was maintained at 40 °C with 1M ammonium acetate in water (as solvent A) and 1M ammonium acetate in propanol (as solvent B). Analysis was performed at a flow rate of 0.6mL/min and monitored at 240 nm.

Cell culture

Murine macrophage RAW cells line. (ATCC® TIB-71™ Rockville, MD, USA) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), in a humidified 5% CO_2 atmosphere at 37 °C.

Determination of nitric oxide (NO) and assessment of cell viability

The toxicity of fruit extracts on RAW 264.7 macrophages was evaluated using the MTT colorimetric method (Scudiero et al., 1988). RAW 264.7 macrophages were seeded in sterile 96-well plates (2×10^4 cells/well) and incubate at 37 °C for 48 hours, medium was removed, and cells were washed with PBS and treated for 30 minutes with different concentrations of

fruit extracts, after which were activated with LPS (1 μg/mL) and incubate again for 24 hours at 37 °C. Following exposure, 70 μL of supernatants were removed and the released NO was measured as its end product, nitrite using the Griess method (Green et al., 1982). Supernatants were mixed with equal volumes of Griess reagent (1% sulfanilamide in 3% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride), the mix was incubated at room temperature for 5 min. Next, the absorbance was measured at 550 nm by a Multiskan EX microplate reader (Thermo Scientific, Waltham, MA, USA). Nitrite concentration was determined by a standard curve prepared with sodium nitrite dissolved in PBS.

On the other hand, the cells were washed with PBS and 100 μL of MTT solution (0.25 mg/mL) was added to each well of the plate. The plates were incubated at 37 ºC for 4 hours in the CO_2 incubator. Finally, supernatant was removed, and 100 μL of DMSO was added to dissolve the formazan crystals and the absorbance was measured at 550 nm in a Multiskan EX microplate reader (Thermo Scientific, Waltham, MA, USA). In each trial, a group of cells not exposed to the extracts was included as a negative control and a group exposed to Triton X-100 (20%) as control of maximum toxicity. The viability percentages were calculated as a percentage, considering the negative control as 100% viability.

Cytokine and prostaglandin E2 (PGE_2)

Raw 264.7 macrophages were plated in 24-well plates (2×10^5 cells/well) and incubated for 48 hours. Cells were pretreated with various concentrations of fruit extracts for 30 minutes and incubated for 24 hours with LPS (1μg/mL). After incubation, the medium was collected. Quantification of TNF-α, IL-6 and IL-1β secreted in the culture medium was made with a commercial enzyme linked immunosorbent assay [ELISA] kit [eBioscience], following the manufacturer's protocol. PGE_2 production was measured using commercially available competitive ELISA kits (Enzo®) according to the manufacturer's instructions.

Western blot analysis

In brief, RAW 264.7 macrophages were collected and dissolved in RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad). Same amount of protein samples (80 μg) was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to nitrocellulose membranes. After, the membranes were blocked with 5% (w/v) dry milk and washed in TBST buffer. The membrane was incubated with specific primary antibody for two hours at room temperature and then incubated with horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. Immunoreactive bands were detected by exposed to X-ray film and their densities were quantified using Carestream Molecular Imaging Software. Western blot data was quantitated to better view the difference between different treatment groups.

Acute toxicity study

Acute oral toxicity study was performed following the protocol by Castro et al. 2015 (Castro et al., 2015) with modifications. Briefly, six- to eight-week-old BALB/c mice (n=6) were selected for the study. The animals fasted overnight with water ad libitum. Golden Berry extract was administered orally at a dose of 2000 mg/kg. The mortality and general behavioral changes were observed for 3 days. For histological analysis, liver and kidney samples were preserved in buffered formalin and stained with hematoxylin and eosin.

Diets and chronic toxicity study

Six- to eight-week-old BALB/c mice were fed with food supplemented with 0,15% or 0,3% Golden Berry extract during 30 days. These percentages were chosen based on the acute toxicity study and that the amount of extract used should reasonably be expected to be achievable in a human population. General behavioral changes were observed for 30 days. For histological analysis, liver and kidney samples were preserved in buffered formalin and stained with hematoxylin and eosin.

Dextran sulfate sodium (DSS)-induced acute colitis.

Colitis was induced employing the method described by Kim et al., 2012, with some modifications (Kim et al., 2012). Briefly, mice were randomly divided into 5 groups (n = 7 per group), control group, DSS alone, DSS + GD (food supplemented with 0,15 and 0,3% of GD extract) and GD alone. All the groups were fed with either commercial mouse food as control group or the same food supplemented with 0,15% or 0,3% GD extract during 30 days and were exposed to 3% DSS in drinking water during the last 9 days. During DSS induced acute colitis, mice were weighed every day and signs of rectal bleeding and diarrhea were monitored. The Disease Activity Index (DAI) was determined according to the parameters described by Li et al., 2014 (L. Li et al., 2014). On the 30th day, mice were sacrificed, the colon was extracted, cleaned, weighed and its length was measured. A colonic tissue sample was preserved in buffered formalin and stained with hematoxylin and eosin or Periodic Acid-Schiff [PAS] for histopathological analysis. The rest of the colon was kept for subsequent determination of ROS level, the MPO activity and cytokines levels.

Measurement of tissue cytokine level

In brief, colon tissue was homogenized in T-PER Tissue Protein Extraction Reagent with protease inhibitor cocktail (Roche), at 4°C using TissueRuptor® (Qiagen, Haan, Germany). Samples were centrifuged at 12.000 rpm at 4°C. The levels of IL-1β, IL-6, IL-10 and TNF-α were quantified on the obtained supernatants with commercial enzyme linked immunosorbent assay [ELISA] kits [eBioscience], following the manufacturer's protocol. The results were

expressed as picogram of cytokine per milligram of protein (pg/mg), which was quantified by the Bradford method, using a standard commercial kit [Biorad 500-0206].

Measurement of tissue ROS level

ROS levels of mice colon were determined using the technique described by Song et al., 2008 (Song et al., 2008). Colon biopsies were frozen in liquid nitrogen and homogenized vigorously in 0.1 M phosphate buffer (pH 7.4), and their homogenate was incubated with DCF-DA (10 mM) for 30 min. Fluorescence intensity was measured in a 96-well Fluoroskan Ascent plate reader (Thermo Scientific, Waltham, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

MPO activity

The enzyme activity was measured according to the technique described by Castro et al. 2019 (Castro et al., 2019), with modifications. In brief, colon tissue was homogenized in phosphate buffer (pH: 7.4) at 4°C using TissueRuptor® (Qiagen, Haan, Germany). Samples were centrifuged at 10.000 rpm at 4°C, the pellet obtained was suspended in a solution of HTAB 0,5% and EDTA 0,3% on phosphate buffer (pH 6.0). The homogenate obtained underwent two fast freeze–thaw cycles, sonication for 10 s and lastly it was centrifuged for 10 min at 10,000 rpm at 4°C. The recovered supernatant was used to evaluate the MPO activity, 50 μL of the supernatant were mixed with 50 μL of O-Dianisidine (0.067%) and 50 μL of hydrogen peroxide (H_2O_2 0.003%). OD_{450} was determined using a Multiskan Go microplate reader (Thermo Scientific, Waltham, MA, USA). The results were expressed as MPO units per milligram of protein; where an activity unit is defined as the amount of enzyme capable of degrading 1 μmol of hydrogen peroxide in a minute at 25°C.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) of three independent experiments. Data were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

In recent years, fruits are attracting growing interest among researchers. This interest is related to the fact that fruits could have the potential to prevent and treat some chronic diseases (Y. Li et al., 2016; Slavin & Lloyd, 2012). Experimental studies demonstrate that modulation of inflammatory response related to intake of fruits is responsible for their positive health outcomes (Pan et al., 2009; Zhu et al., 2018). In this work we evaluated the anti-inflammatory

potential *in vitro* of the ethanol extracts of ten fruits: yellow pitaya, banana passion, purple passion fruit, goldenberry, tamarillo, lulo, soursop, kalipatti sapota, sapote and sour guava; and the most activity fruit extracts was selected to be evaluated in an animal model of inflammatory bowel diseases. The information of fruits selected for the study are in Table 6.1.

Table 6.1. Information on fruits included in this study.

Fruits	Part used	Lyophilized pulp yields (%)	Extract yields (%)
Yellow pitaya *Hylocereus megalanthus*	pulp and seed	24,54	30,08
Banana passion *Passiflora cumbalensis*	pulp	9,78	81.41
Purple passion fruit *Passiflora edulis*	pulp	13,04	84,44
Golden berry *Physalis peruviana*	Whole fruit	18,08	44,70
Tamarillo *Solanum betaceum*	pulp	9,10	42,87
Lulo *Solanum quitoense*	pulp and seed	5,46	60,0
Soursop *Annona muricata*	pulp	13,12	57,91
Kalipatti sapota *Manilkara zapota*	pulp	32,27	49,95
Sapote *Pouteria sapota*	pulp	26,26	36,21
Sour guava *Psidium friedrichsthalianum*	pulp	12,01	52,66

Effect of fruits extracts on ·NO, IL-1β, IL-6, TNF-α and PGE-2 production in LPS-activated RAW 264.7 macrophages

Human clinical studies suggest that higher fruit intake is associated with reduced levels of common inflammatory biomarkers, including CRP, IL-1β, IL-6 or TNF-α, for different population and age groups (Wu & Schauss, 2012). With the exception of CRP, these inflammatory biomarkers is mostly secreted by macrophages. Therefore, identify fruits that can inhibit the production of these inflammatory mediators in macrophages is a good starting point for prevention and treatment of many chronic diseases.

Even though ·NO is not one of the common inflammatory biomarkers, it is an important inflammatory mediator, since the inhibition of iNOS activity or down-regulation of iNOS expression is desirable to reduce the extent of inflammatory response (Zhu et al., 2018). In addition, it has the advantage of being easily quantifiable and at a very low cost, considered as being a suitable method for the screening of plant species as a strategy in the search for metabolites with anti-inflammatory activity.

In this work, first we evaluated the anti-inflammatory potential of ten fruit extracts, determining their capacity to inhibit the production of the inflammatory mediator nitric oxide in macrophages RAW 264.7, activated with LPS (Figure 6.1). It can be observed the extracts

of purple passion fruit, yellow pitaya, tamarillo and goldenberry were the most active extracts, with inhibition percentages higher than 20%. All extracts were evaluated at non-toxic concentrations for macrophages, which were determined using the MTT test (data not shown).

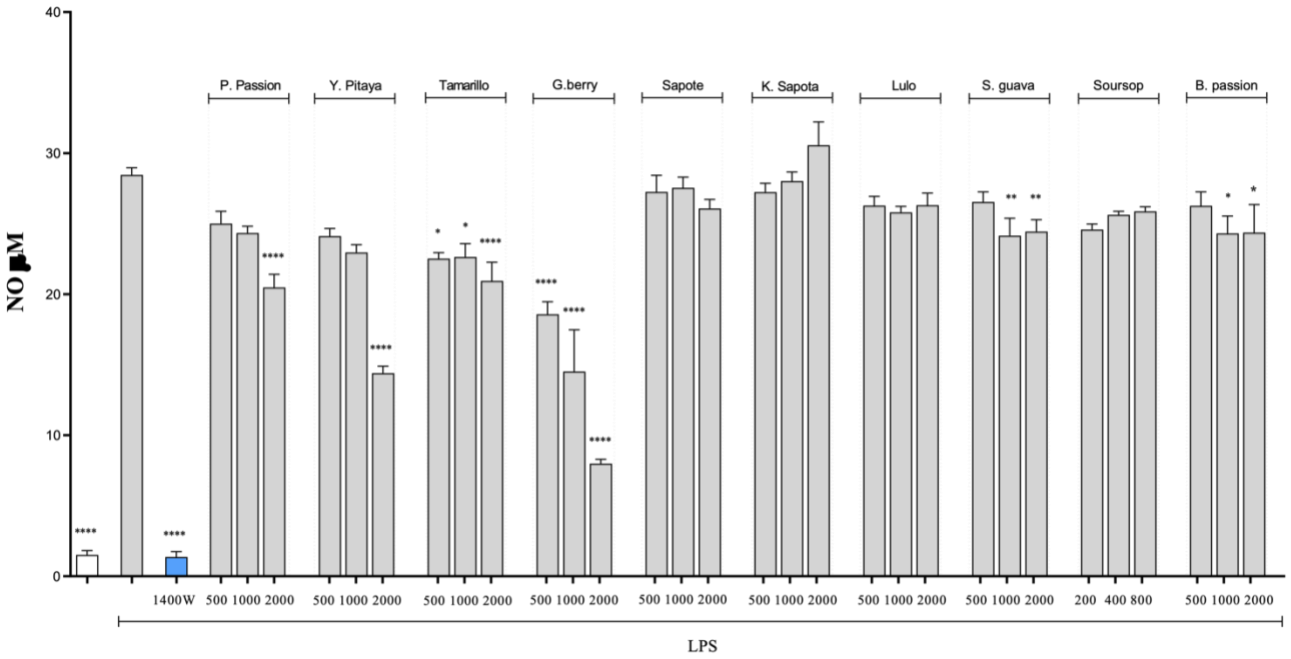

Figure 6.1: Effect of extracts on cell viability and production of NO, in LPS-stimulated RAW 264.7 macrophages (1µg/mL) for 24 h. 1400W (10 µM) was used as positive control. The results represent the mean ± standard error of the mean. (*p<0,01, **p<0,001 and ****p < 0.00001 statistically significant compared with LPS treated group).

The effect of the most active extracts on the inflammation biomarkers IL-1β, IL-6, TNF-α and PGE-2 was determined, whose decrease could prevent or suppress a variety of inflammatory diseases (Zhu et al., 2018). Results show that all evaluated extracts except the extract of purple passion significantly decreased the production of IL-6 and IL-1β, being the most active the extract of goldenberry (Figure 6.2 A and B). Regarding the effect on TNF-α, all the extracts significantly inhibited the production of this mediator and again the extract of goldenberry presented the best inhibitory activity (Figure 6.2 C). Finally, the results presented in Figure 6.2 D show that only the extracts of goldenberry and tamarillo inhibited the production of PGE2 significantly. In other study extracts of goldenberry has shown immunomodulatory effects of the expression of inflammatory biomarkers on human cervical cancer (HeLa), murine fibroblast (L929) cells and in liver (Mier-Giraldo et al., 2017; Pino-De la Fuente et al., 2020).

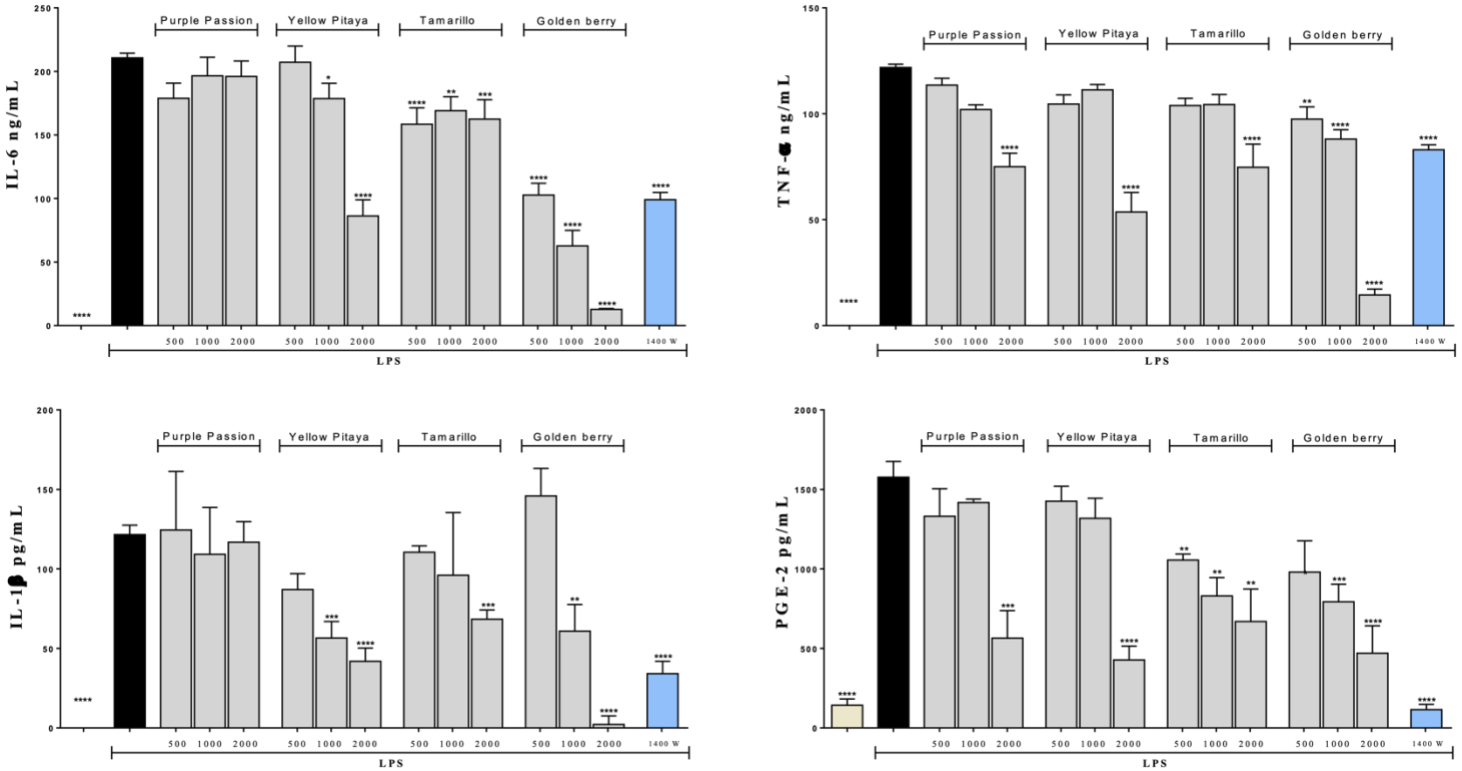

Figure 6.2: Effect of the extracts of purple passion, yellow pitaya, tamarillo and golden berry on cell viability and production of IL-6, IL-1β, TNF-α and PGE-2, in LPS-stimulated RAW 264.7 macrophages (1μg/mL) for 24 h. 1400W (10 μM), Dexamethasone (20 μM) and Rofecoxib (10 μM) were used as positive control. The results represent the mean ± standard error of the mean. (**p*<0,01, ***p*<0,001, ****p* < 0.0001 and *****p* < 0.00001 statistically significant compared with LPS treated group).

Western blot

The modulation of inflammation by phytochemicals present in fruits has been associated with the inhibition of several transcription factors, among which is NF-κB (Iddir et al., 2020; Oveissi et al., 2019). The nuclear transcription factor NF-κB is a ubiquitous factor that resides in the cytoplasm. When activated by various stimuli such as the action of LPS on the TLR4 receptor, it translocates to the nucleus, due to phosphorylation and proteolytic degradation of the inhibitory protein IκBα, an endogenous inhibitor that binds to the transcription factor NF-κB in the cytoplasm and its Degradation allows transcription factor NF-κB to move to the nucleus, where it binds to promoters that induce the coordinated expression of many genes that encode cytokines, chemokines, enzymes, and adhesion molecules involved in the amplification and perpetuation of the inflammatory reaction. Therefore, inhibition of transcription factor NF-κB is generally considered a useful strategy for the treatment of inflammatory disorders and this pathway represents an important and highly attractive therapeutic target for compounds that selectively interfere with it (Niu et al., 2015; Yoon & Baek, 2005).

To confirm whether the inhibitory effect of cape gooseberry extract on NO, IL-6, IL-1β, TNF-α, and PGE-2 was related to the inhibition of the transcription factor NF-κB, we examined the effects of extracts of goldenberry on the degradation of IκB in LPS-stimulated macrophages. As shown in Figure 6.3, the level of IκB protein in the cytoplasm decreased by LPS treatment, indicating IκBα degradation. However, the decreased level of IκB protein in the cytoplasm was markedly increased by the extracts of goldenberry, implying the extracts of goldenberry prevents NF-κB activation. We further examined the effect of the extracts of goldenberry on NF-κB activation and translocation of NF-κB p65 from the cytosol to the nucleus. Further, LPS stimulation caused translocation of p65 from the cytosol to the nucleus, while treatment with the extracts of goldenberry reduced this translocation (Figure 6.3). Previous studies have shown that compounds isolated from cape gooseberry have significantly inhibited the activity of the transcription factor NF-κB in HEK 293 / NF-kB-Luc cells (Chang et al., 2016).

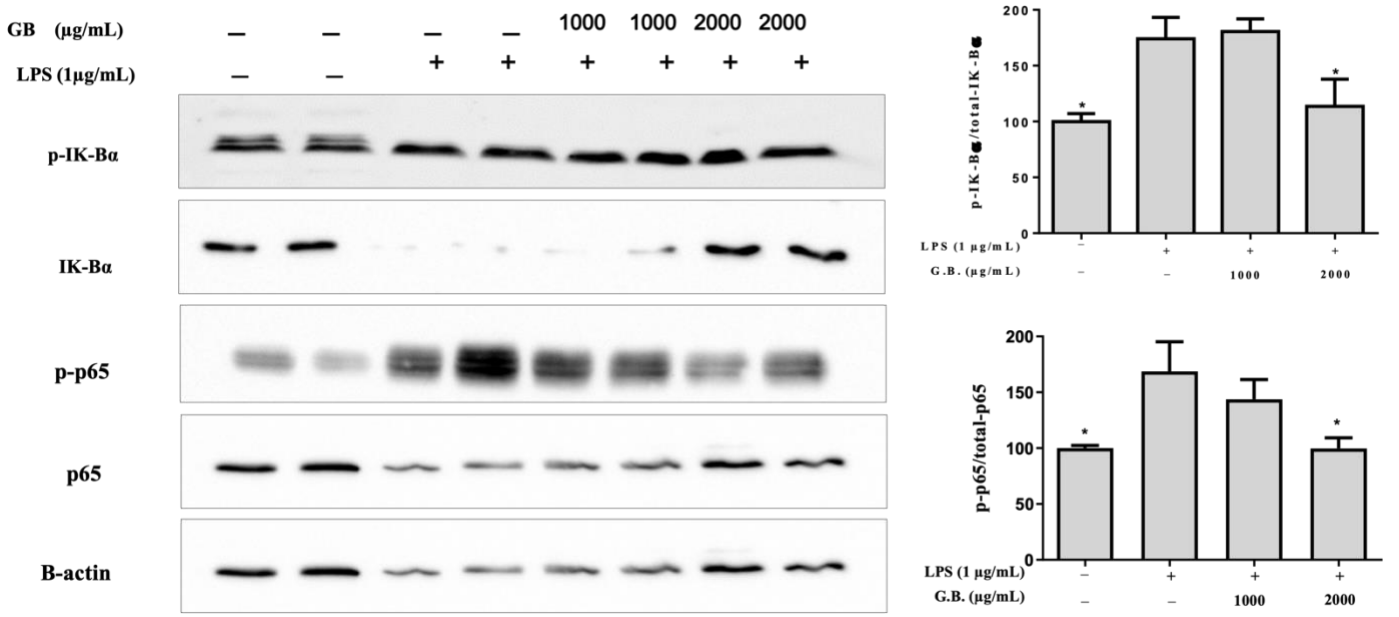

Figure 6.3: Inhibition of the nuclear transcription factor NF-κB by the extracts of golden Berry in LPS-stimulated RAW 264.7 macrophages (1μg/mL) for 24 h.The data shown are representative of three independent experiments.

Goldenberry anti-inflammatory effect on mouse with inflammatory bowel disease

Nutrition plays a complex role in the etiology of inflammatory bowel disease (IBD). Still, it is difficult to establish the interaction of nutrients, host genetics, intestinal microbiota, immune response, and IBD risk (Gentschew & Ferguson, 2012; Hou et al., 2011). The importance of diet in the prevention and/or development of IBD is since diet not only has a direct effect on inflammation of the intestine through the regulation of inflammatory mediators, but it can also influence epigenetic modifications and the intestinal microbiota (Spooren et al., 2013).

Certain food products, including fruits, can regulate the immune system's response and modify intestinal inflammation (Bernstein, 2017; Salaritabar et al., 2017). The fruit of the *Physalis peruviana* L. species, commonly known as cape gooseberry, is used in traditional medicine to lower blood sugar levels, to prevent the formation of cataracts, and to treat intestinal problems such as ulcers and diarrhea (John James Rodríguez-Echeverry, 2010; Toscano Gonzalez, 2006). There are many studies in the literature on various activities of the Physalis peruviana fruit, including reports of anti-inflammatory activity (Chang et al., 2016; Hassan et al., 2017; Mier-Giraldo et al., 2017).

Taking into account the anti-inflammatory history of cape gooseberry and the results found in this study, whereof the ten fruits studied, it proved to have the best anti-inflammatory potential, by significantly inhibiting the production of NO, IL 6, IL-1β, TNF α and PGE-2, in LPS-stimulated macrophages, we decided to evaluate the immunomodulatory effect of a diet supplemented with 0,15% and 3% golden berry fruit extract on mice with DSS-induced inflammatory bowel disease. These percentages were chosen based on the acute toxicity study and that the amount of extract used should reasonably be expected to be achievable in a human population. The animals did not show signs of toxicity during the 72 hours after being administered with a dose of 2000 mg / Kg of the extracts of golden berry. The results of the histological analysis also revealed that there was no damage to the liver or kidney (data not shown).

Inflammatory bowel disease is characterized by weight loss; therefore, the animals were weighed during the 30 days of the trial. The animals that consumed a diet supplemented with currant extract presented less weight loss than those of the sick control group. Being, the group with food supplemented to 0.15% which lost less weight (Figure 6.4A). On the other hand, the disease activity index was determined, which was given under a calculated score according to the variation in weight, the consistency of the stool, and the bleeding in the stool. The sick control group obtained a higher score (Figure 6.4B)

On day 30 of the test, the animals were sacrificed, and macroscopic analysis of the colon was carried out, measuring the length of the colon and calculating the weight/length ratio of the colon; this ratio indicates the degree of damage that occurs in the intestinal mucosa. As expected, the healthy control had a normal appearance, and the length of the colonic tissue was greater than the group with colitis induced by sodium dextran sulfate (DSS). The groups to which the food supplemented with 0.15 and 0.3% of the cape gooseberry extract were administered presented a greater length of the colon than the group to which only DSS was administered, especially the group that consumed the food supplemented at 0.15%. (Figure 6.4C and D).

Regarding the results of the weight/length relationship, it is observed that there is a large increase in this relationship in the group with colitis induced by sodium dextran sulfate (DSS)

because this agent caused lesions that affected the intestinal mucosa and submucosa; while in the groups treated with cape gooseberry extract, the index was reduced, indicating an improvement in inflammatory bowel disease. The foregoing evidenced that the cape gooseberry extract presented an anti-inflammatory effect since it allowed the colon to shorten to a lesser extent in the treated groups, indicating a lower degree of inflammation. (Figure 6.4E).

The results of the macroscopic analysis correspond to the histological findings. The groups in which IBD was induced presented ulceration, edema, fibrosis, inflammation, neutrophil infiltration, and increased thickness of the submucosal layer and the muscle layer; However, it is important to note that in the group treated with the food supplemented with 0.15% gooseberry extract, restoration was observed in the colonic tissue and the infiltration of neutrophils was reduced. (Figure 6.4F and G). The healthy group and the one that received only the 0.3% supplemented diet were in normal conditions and without alteration in the cells.

On the other hand, using the PAS staining technique, it was observed that the group treated with food supplemented with 0.15% gooseberry extract despite presenting goblet cell depletion, these were lower than those presented in diseased animals that received the food supplemented at 0.3% and the animals treated only with DSS. It was also observed that the group that only had food supplemented at 0.3% did not present significant differences with the healthy group, indicating that the extract is not responsible for the depletion of goblet cells (Figure 6.4F and H).

The mechanisms of the intestinal anti-inflammatory effect of dietary supplementation with gold berry extract can be associated with a decrease in the activity of the MPO enzyme and the production of ROS, IL-6, IL-1β, IL-10, and TNF-α, in the colon tissue. However, the diet supplemented with cape gooseberry only managed to significantly decrease IL-6 and IL-1β production (Figure 6.5A and B), the activity of the MPO enzyme and the levels of ROS, IL-10, and TNF-α also decreased (Figure 6.5C, D, E, and F); the simultaneous decrease of all these mediators may have had an important impact on the immunomodulatory effect shown by cape gooseberry extract in mice with DSS-induced inflammatory bowel disease.

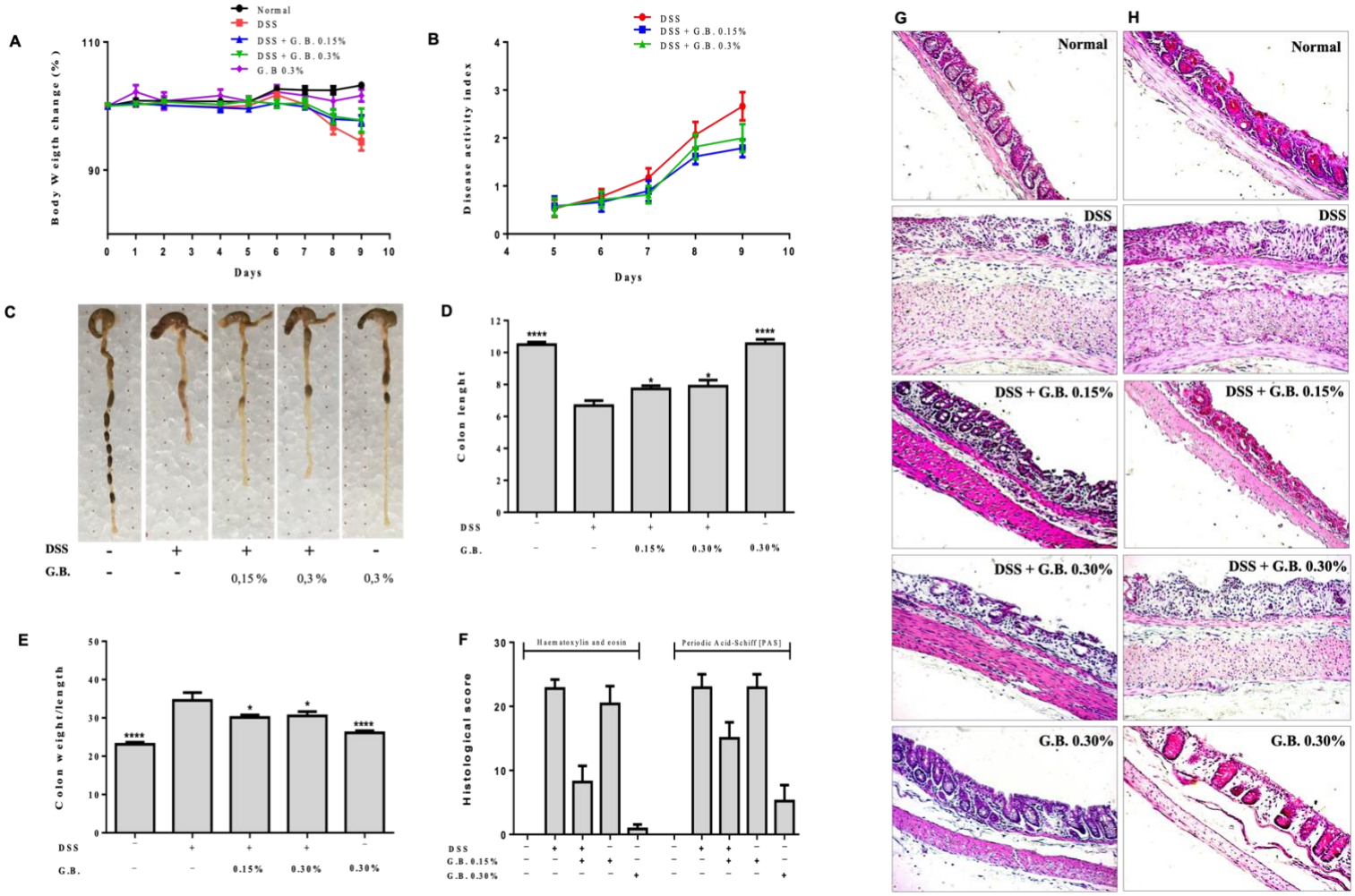

Figure 6.4: Golden berry dietary supplementation attenuates pathological symptoms of Dextran Sulfate Sodium (DSS) induced acute colitis. BALB/c mice were fed with golden berry (0.15 y 0.3 %) for 30 days and exposure to 3% DSS in drinking water during the last 10 days. A: Body weight changes following the DSS colitis induction. B: Disease activity index. C y D: Macroscopic analysis (measuring the length of the colon) E: calculating the weight / length ratio of the colon. F and G: measuring the length of the colon and calculating the weight/length ratio of the colon. F and H: Crypt loss and goblet cells depletion were determined by periodic acid Schiff [PAS]-staining. The results represent the mean ± SEM. [n = 10]. (*$p<0,01$ and ****$p < 0.00001$ statistically significant compared with DSS group).

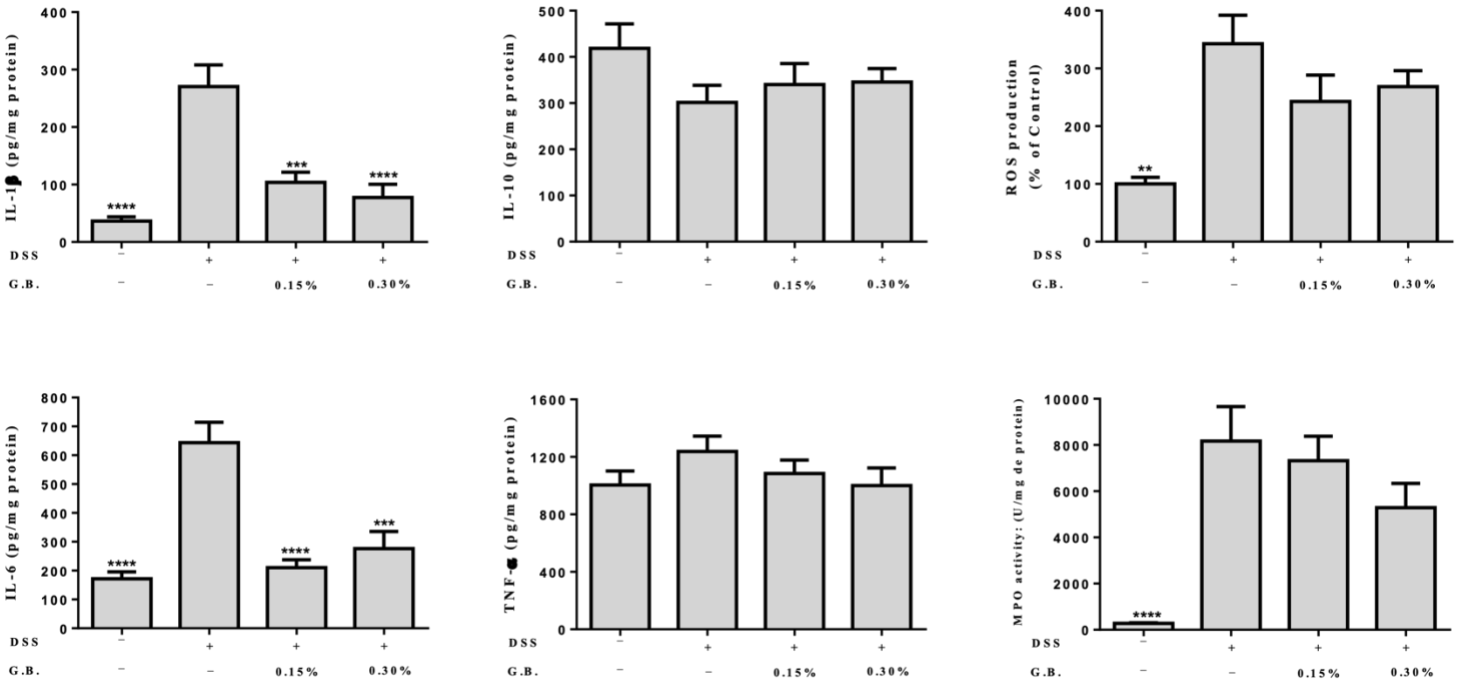

Figure 6.5. Golden berry dietary supplementation effect on Myeloperoxidase [MPO] enzyme activity and IL-1β, IL-6, IL-10, TNF-α and ROS levels in colonic tissue. The results represent the mean ± standard error of the mean [SEM] [n = 10]. (**p<0,001, ***p < 0.0001 and ****p < 0.00001 statistically significant compared with DSS group).

CONCLUSION

In conclusion, our results demonstrate that dietary supplementation with gold berry extract attenuates the pathological symptoms of acute colitis induced by sodium dextran sulfate (DSS) in BALB / c mice. Golden berry extract reduces bleeding, weight loss, diarrhea and shortening of the colon. The mechanisms of intestinal anti-inflammatory effect of golden berry can be associated with the inhibition of MPO enzyme activity and the decrease in production of IL-6, IL-1β, IL-10, TNF-α, and ROS. In summary, the ingesting of golden berry as a functional food in the diet might contribute to the treatment of inflammatory bowel disease.

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CAPÍTULO 3: Discusión general

Desde tiempos inmemoriales, las plantas se han utilizado no solo como alimento sino también como fuente de sustancias biológicamente activas. El uso de plantas con fines medicinales se documentó en manuscritos sumerios hace más de 5000 años. Las sustancias de origen vegetal fueron durante mucho tiempo la única fuente de medicamentos e incluso en la actualidad, estas sustancias se utilizan ampliamente en la práctica sanitaria, debido a la inmensa variedad de metabolitos secundarios funcionalmente relevantes presentes en las especies vegetales (Dushenkov & Raskin, 2008)

Según estimaciones de la Organización Mundial de la Salud (OMS), alrededor del 80% de la población de los países en desarrollo dependen de preparaciones vegetales para el tratamiento de sus enfermedades, aunque también son ampliamente utilizadas en los países desarrollados como se evidencia en la gran cantidad de preparaciones vegetales descritas en las farmacopeas de Japón, Alemania y otros países europeos. (Dushenkov & Raskin, 2008)

La diversidad química del reino vegetal permanece en gran parte inexplorada. La flora de nuestro planeta es una enorme fuente de perspectivas futuras para la bioterapia y la mejora de la atención de la salud pública. Colombia cuenta con una enorme diversidad biológica, la cual incluye entre 45 a 50 mil especies de plantas, lamentablemente para un elevado porcentaje de estas especies se cuenta con un pobre conocimiento, lo que ha evitado aprovechar los beneficios que puede aportar esta flora en la alimentación, la medicina y en la industria, mientras se está deteriorando su valor estético natural y su aporte al equilibrio global (Tobasura, 2006).

Una vía alternativa para aprovechar en mayor medida estos recursos biológicos es la práctica de la bioprospección, la cual implica la interacción entre diferentes tipos de conocimiento, especialmente aquel conocimiento ancestral con los que cuentan las comunidades locales e indígenas de los países sudamericanos y el conocimiento científico o tecnológico aportado por los sectores académicos e investigativos y las actividades comerciales propias del sector industrial especialmente la industria farmacéutica. Para esta industria, Colombia tiene un enorme atractivo para emprender prácticas de bioprospección, debido a su gran biodiversidad, que representa una enorme fuente de compuestos químicos y biológicos novedosos con gran potencial de comercialización; pero desafortunadamente se cuenta con muy pocos estudios que contribuyan a cuantificar este potencial (Duarte & Velho, 2010).

Los preparados a base de plantas para el tratamiento de las enfermedades y los trastornos de salud más comunes, son parte integral de la cultura colombiana, sin embargo, muchas de las especies vegetales empleadas se usan solo por tradición, ya que como se planteó anteriormente en este documento no cuentan con ningún estudio científico que respalde su uso. Esta tesis doctoral aporta información que permite corroborar con una base científica el

uso dado a algunas especies vegetales en la medicina popular de Colombia para tratar enfermedades que cursan con procesos inflamatorios y/o asociados al estrés oxidativo.

En esta tesis se estudió el potencial anti-inflamatorio de 51 especies vegetales utilizadas en la medicina popular de Colombia. Los resultados evidenciaron numerosos extractos con actividad anti-inflamatoria entre los que se resaltan los extractos etanólicos de las semillas de *Ambrosia peruviana*, las hojas de *Mammea americana*, los cálices de *Physalis angulata* y el fruto de *Physalis peruviana*; los cuales inhiben significativamente la producción del mediador inflamatorio NO•. Estos resultados se consideran muy prometedores teniendo en cuenta que se trata de uno de los biomarcadores de inflamación más importantes en los procesos inflamatorios asociados a las ECNT. En función de estos resultados se procedió a evaluar el efecto de estos extractos sobre la producción de otros importantes mediadores del proceso inflamatorio, encontrándose que también eran capaces de disminuir significativamente los niveles de IL-1β, IL-6, TNF-α y PGE-2; en macrófagos RAW 264.7 estimulados con LPS. Adicionalmente, estos extractos también mostraron una prometedora actividad antioxidante, con capacidad para captar diversos radicales como: DPPH, ABTS, peroxilo y óxido nítrico. Estos resultados evidencian claramente el potencial anti-inflamatorio y antioxidante de los extractos obtenidos de las hojas de *Mammea americana*, los cálices de *Physalis angulata*, y el fruto de *Physalis peruviana.* En este orden de ideas y con miras a darle continuidad a estos resultados y evidenciar su comportamiento en modelos *in vivo*, se evaluó el efecto anti-inflamatorio del extracto total etanólico de las hojas de *Mammea americana* y los cálices de *Physalis angulata* en un modelo de edema auricular inducido por TPA. Adicionalmente, también se evaluó el potencial anti-inflamatorio del extracto etanólico total de las flores de *Cordia alba*, en el mismo modelo, siendo estos resultados el primer reporte del potencial antiinflamatorio de las flores de esta especie. Mientras que el extracto del fruto de la especie *Physalis peruviana* fue evaluado en un modelo de enfermedad inflamatoria intestinal.

Mammea americana, conocido comúnmente como Mamey, es un árbol de hoja perenne, que se ha clasificado como perteneciente a la familia Calophyllaceae. Es nativo de las Indias Occidentales y del norte de Sudamérica. Comúnmente se cultiva por su fruto en las llanuras y estribaciones de la costa del Pacífico, crece en Colombia, Venezuela, Guyana, Surinam, Ecuador y el norte de Brasil (Yahia & Guttierrez-Orozco, 2011). No se cuentan con estudios de mamey para actividad anti-inflamatoria y los reportes relacionados con propiedades antioxidante son limitados; se encuentran algunos estudios como el de la evaluación de su capacidad captadora de radicales usando el ensayo de radicales libres DPPH (1,1-difenil-2-picrilhidrazilo) (González-Stuart, 2011), o el estudio realizado por Toma, Hiruma-Lima *et al.* 2005.

Ambrosia peruviana, es una planta perenne, aromática, de hasta 2 m de altura, hojas alternas u opuestas, cubierta en el fondo de vellosidades largas, nativa de América Central, donde se

conoce como "Altamisa" y/o "Artemisa". Sus flores masculinas son verdes en los capítulos terminales, mientras que las flores femeninas se agrupan en las axilas superiores de las hojas; los frutos son ovoides, angulosos y espinosos. En la medicina tradicional, *Ambrosia peruviana* se usa en varios países de América del sur para el tratamiento de cólicos, sangrado, dolor crónico, artritis, espasmos, infecciones, entre otras afecciones. Los estudios fitoquímicos de *Ambrosia peruviana* han sido dirigidos principalmente a la extracción, aislamiento y elucidación estructural de lactonas sesquiterpénicas, reconocidas principalmente por su actividad citotóxica (Cicció & Chaverri, 2015; Gómez-Estrada et al., 2011; Jimenez-Usuga et al., 2016; Rodríguez Alviz et al., 2014).

En el artículo "Anti-inflammatory screening of plant species from the Colombian Caribbean Coast", el cual hace parte del desarrollo de esta tesis doctoral se demostró que tanto el extracto etanólico de hojas de *Mammea americana* como el de las semillas de *Ambrosia peruviana* inhibieron significativamente la producción de importantes marcadores inflamatorios *in vitro*, usando macrófagos RAW 264.7, estimulados con LPS. Además, mostraron una elevada capacidad para capturar diversos radicales libres y disminuir los niveles de EROs en los macrófagos estimulados. Tanto el extracto de hojas de *Mammea americana* como el extracto de semillas de *Ambrosia peruviana* lograron inhibir los niveles de los mediadores inflamatorios: NO, TNF-α, IL-6 e IL-1β (Jenny P. et al., 2021). El potencial anti-inflamatorio del extracto de las hojas de *Mammea americana*, pudo ser corroborado *in vivo*, este extracto disminuyó el edema auricular inducido por el TPA e inhibió la actividad de la enzima MPO, usada como marcador de la infiltración de neutrófilos polinucleares al sitio de la inflamación.

Physalis angulata conocida en Colombia como Topo-Toropo es un arbusto pequeño de la familia Solanaceae que ha sido ampliamente estudiado y del cual se han aislado gran cantidad de compuestos, principalmente Witanólidos y otros compuestos de tipo esteroidal denominados "Physalins", los cuales han mostrado potente actividad anticancerígena, antibacteriana, antidiabética, anti-malaria, antianémica, antipirética y anti-inflamatoria (Rengifo-Salgado & Vargas-Arana, 2013). Sin embargo, a pesar de la enorme atención que ha despertado esta planta, con numerosos estudios y la gran cantidad de moléculas bioactivas aisladas de sus órganos, los cálices, formados por sépalos o pequeñas hojas modificadas encargadas de cubrir y proteger a la fruta formando una bolsa en forma de farol; han sido poco estudiados (Kindscher et al., 2012; Rengifo-Salgado & Vargas-Arana, 2013). Los resultados del efecto anti-inflamatorio de los cálices de *P. angulata* obtenidos en la ejecución de esta tesis doctoral y publicados en el artículo "A screening of plants used in Colombian traditional medicine revealed the anti-inflammatory potential of *Physalis angulata* calyces" (Rivera et al., 2019), constituyen un importante aporte al conocimiento de las propiedades farmacológicas de esta especie, en donde el extracto total de los cálices de *P. angulata* y su fracción en diclorometano inhibieron significativamente la inflamación en el tejido auricular,

y esta actividad parece estar relacionada con la capacidad de inhibir la producción de NO, PGE-2, IL-1β, IL-6, TNF-α y la proteína quimiotáctica de monocitos (MCP) -1 y (CCL2).

Cordia alba (Jacq.) Roem y Schult. es nativa del Sur y Centroamérica (Cervantes Ceballos et al., 2017). Las flores de esta especie son ampliamente utilizadas en la Costa Caribe colombiana para tratar problemas inflamatorios. Los estudios *in vitro* mostraron que el extracto de flores de *Cordia alba* inhibía la producción de NO en macrófagos RAW 264.7 estimulados con LPS. A pesar de que no se observó una potente inhibición de este mediador, la moderada actividad observada sumada al hecho de que estos resultados representan el primer informe científico que respalda el uso tradicional como anti-inflamatorio de las flores de esta especie vegetal, se procedió a evaluar la actividad de este extracto en el modelo *in vivo* de edema auricular inducido por TPA. Los resultados de esta evaluación muestran que el extracto etanólico total de flores de *Cordia alba* disminuye significativamente el edema causado por el TPA, así como la actividad de la MPO en el tejido auricular. Además, mostró importantes efectos captadores de los radicales libres DPPH y ABTS.

Physalis peruviana L., perteneciente a la familia Solanaceae, es una planta herbácea, semi-arbustiva, erecta y perenne en zonas subtropicales, puede crecer hasta alcanzar 0.6 o 0.9 m o incluso hasta 1.8 m. Entre las partes de esta planta están: las hojas, el tallo, la flor; la cual es fácilmente polinizable. El fruto es una baya jugosa con forma ovoide y un diámetro entre 1.25 a 2.50 cm, 4 y 10 g peso (Puente et al., 2011) el cual es conocido popularmente como uchuva en Colombia, uvilla en Ecuador, aguaymanto en Perú, topotopo en Venezuela y goldenberry en países de habla inglesa; el fruto de *Physalis peruviana* L., ha sido usado como fuente de provitamina A, minerales, vitamina C y complejo vitamínico B. Contiene 15% de sólidos solubles (principalmente azúcares). Además, contiene; fósforo, calcio, hierro, ácido ascórbico, hidratos de carbono, y un alto contenido de fibra dietética, que le permite actuar como regulador intestinal (Ramadan, 2011).

La actividad anti-inflamatoria de uchuva ha sido corroborada por varios estudios en su mayoría a nivel *in vitro*. En el estudio realizado por Mier-Giraldo, *et al.* 2017, extractos del fruto de uchuva inhibieron la producción de IL-6 y IL-8 en células HeLa (Mier-Giraldo et al., 2017). En otro estudio se encontró que la administración de jugo de uchuva disminuía la producción de TNF-alfa en el hígado de ratas con carcinoma hepatocelular (Hassan et al., 2017). Compuestos aislados de uchuva han inhibido significativamente la actividad del factor de transcripción NF-κB en células HEK 293/NF-kB-Luc (Chang et al., 2016).

Como un aporte adicional, en este proyecto se demostró en ensayos realizados con macrófagos RAW 264.7 estimulados con LPS que el extracto del fruto de la especie *Physalis peruviana* disminuyó significativamente la producción de NO, IL-6, PGE-2, ROS, IL-1β y TNF-α; mediadores importantes en el proceso inflamatorio crónico y/o el estrés oxidativo, además inhibió la activación del factor de transcripción NF-kB. Por otro lado, este extracto

también mostró una importante actividad captadora de los radicales libres DPPH, ABTS, NO, peroxilo. Además de capturar el peróxido de hidrógeno un importante precursor de radicales libres y un importante poder reductor mediante el método FRAP (Mejia et al., 2020).

Considerando la potente actividad anti-inflamatoria *in vitro* mostrada por el extracto obtenido de los frutos de *Physalis peruviana,* la cual constituye una fruta de amplio consumo tanto en el mercado nacional como internacional, donde Colombia es uno de los mayores productores en el mundo, y teniendo en cuenta que el consumo periódico de algunos fitoquímicos puede modular el proceso inflamatorio crónico y el estrés oxidativo, se planteó la hipótesis de que el suministro de una dieta suplementada con el extracto de uchuva podría modular la respuesta inflamatoria en un modelo de colitis aguda en ratones. Para ello se evaluó el efecto del extracto en el modelo agudo de colitis ulcerosa inducida por dextrán sulfato de sodio (DSS) en ratones BALB/c; los resultados de esta evaluación muestran que la dieta suplementada con extracto del fruto de *Physalis peruviana* atenuó los síntomas patológicos de la colitis inducida por el DSS, reduciendo el sangrado, la pérdida de peso, la diarrea y el acortamiento del colon. Adicionalmente se evaluó el efecto del extracto sobre la liberación de importantes mediadores inflamatorios, los resultados sugieren que el efecto anti-inflamatorio parece estar asociado a efectos sobre la migración de polimorfonucleares y la disminución de la producción de IL-6, IL-1β, IL-10, TNF-α y ROS en el tejido colónico.

Estos resultados indican que la ingesta regular del fruto de *Physalis peruviana* en la dieta podría tener un efecto inmunomodulador en el colon inflamado pudiendo ser de utilidad como coadyuvante en el tratamiento de la enfermedad inflamatoria intestinal. Sin embargo, es necesario la realización de posteriores estudios encaminados a profundizar en los mecanismos de acción anti-inflamatorio y antioxidante que pueda tener este fruto al ser consumido en la dieta, así como estudios clínicos que demuestren la seguridad y efectividad del extracto. Lo cual finalmente conduciría al desarrollo de alimentos funcionales a base de estas frutas con el fin de proponer alimentos que afecten positivamente en el tratamiento de enfermedades crónicas no transmisibles asociadas a procesos inflamatorios y oxidativos.

Conclusiones finales

En resumen, el desarrollo de esta tesis doctoral permitió identificar cinco (5) especies vegetales con actividad inhibitoria de importantes biomarcadores del proceso inflamatorio asociado a enfermedades crónicas no transmisibles. Lo cual se evidencia en el cuerpo de esta tesis doctoral y en los artículos científicos que la soportan. A continuación, se describen los resultados más relevantes de este trabajo:

1. Se identificaron en las especies *Ambrosia peruviana*, *Mammea americana*, *Physalis angulata*, y *Physalis peruviana* extractos y fracciones con potente actividad

inhibitoria sobre la producción de importantes mediadores del proceso inflamatorio en modelos *in vitro*.

2. Se corroboró la actividad anti-inflamatoria del extracto de *Mammea americana*, y la fracción primaria en diclorometano de *Physalis angulata* en el modelo *in vivo* de edema auricular en ratones, profundizando en los mecanismos de acción relacionados.

3. Se reportó por primera vez en la literatura actividad antinflamatoria para el extracto etanólico total de las flores de *Cordia alba.*

4. Se identificaron frutos tropicales con capacidad de modular la producción de mediadores inflamatorios y el estrés oxidativo en modelos *in vitro*, los cuales podrían prevenir la aparición de enfermedades crónicas no transmisibles o actuar como coadyuvantes en el tratamiento de las mismas.

5. Se demostró la efectividad de una dieta suplementada con el extracto del fruto de *Physalis peruviana* para modular la respuesta inflamatoria en un modelo químico de enfermedad inflamatoria intestinal en ratones.

Desafíos y perspectivas futuras

Los importantes hallazgos de este trabajo deben continuar siendo explorados con miras a la obtención de productos farmacéuticos o alimenticios basados en estas especies vegetales, útiles para la prevención o tratamiento de enfermedades crónicas no transmisibles asociadas a procesos inflamatorios. Para lo cual es necesario profundizar en los siguientes aspectos:

1. Caracterizar los extractos mediante la obtención de perfiles cromatográficos por HPLC que permitan identificar componentes claves para garantizar la reproducibilidad en la posterior obtención de los extractos desde el material fresco.

2. Realizar estudios de preformulación de los extractos y fracciones donde se evalúen los principales factores que afectan la estabilidad física, química y farmacológica de estos.

3. Realizar estudios toxicológicos de estas especies vegetales de acuerdo a lo establecido por la OECD, que permita garantizar su uso por la población de manera segura.

4. Proporcionar información suficiente sobre los criterios de seguridad y eficacia de estas especies vegetales para solicitar su inclusión en el listado de plantas medicinales para productos fitoterapéuticos, en la categoría preparaciones farmacéuticas con base en plantas medicinales.

5. Realizar estudios clínicos que permitan evidenciar el efecto anti-inflamatorio declarado y su acción sobre biomarcadores del proceso inflamatorio.

La ejecución de estas actividades permitirá el desarrollo, fabricación y comercialización de fitopreparados y/o alimentos funcionales, útiles en la prevención y/o tratamiento de enfermedades crónicas no transmisibles.

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APENDICÉS

Apéndice 1. Publicaciones

- Jenny Castro, Luis Franco, Fredyc Diaz: Anti-inflammatory screening of plant species from the Colombian Caribbean Coast. Journal of Applied Pharmaceutical Science Vol. 11(04), pp 106-117, April, 2021
  DOI: 10.7324/JAPS.2021.110413
  Impact Factor 2021: 0.62. (Q2)

- David Rivera, Yanet Ocampo, Jenny Castro, Lía Barrios, Fredyc Diaz, Luis Franco: A screening of plants used in Colombian traditional medicine revealed the anti-inflammatory potential of *Physalis angulata* calyces. Saudi Journal of Biological Sciences 26 (2019) 1758–1766.
  DOI: 10.1016/j.sjbs.2018.05.030
  Impact Factor 2021: 2.802 (Q1)

- Jenny Castro, David Rivera, Luis A. Franco: Topical anti-inflammatory activity in TPA-induced mouse ear edema model and *in vitro* antibacterial properties of *Cordia alba* flowers. Journal of Pharmaceutical Investigation (2019) 49:331–336
  DOI: 10.1007/s40005-018-00421-z
  Impact Factor 2021: 3.22 (Q1)

- Nely Mejia, Jenny Castro, Yanet Ocampo, Ruben Salas, Carla Delporte, Luis Franco: Evaluation of antioxidant potential and total phenolic content of exotic fruits grown in Colombia. Journal of Applied Pharmaceutical Science Vol. 10(09), pp 050-058, September, 2020.
  DOI: 10.7324/JAPS.2020.10906
  Impact Factor 2021: 0.62 (Q2)

Apéndice 2. Eventos científicos

1. XX Congreso Nacional de estudiantes de Química Farmacéutica. Barranquilla Colombia. Noviembre 2019. Evaluación *in vitro* del potencial antioxidante de 12 extractos de frutas cultivadas en Colombia, sobre la línea celular macrófagos RAW 264.7 estimulados con LPS.

2. World Conference on Pharmaceutical Chemistry and Clinical Research. Valencia-España. Noviembre 2019. *In vivo* anti-inflammatory effect of the total ethanolic extract from the leaves of *Mammea americana,* using a TPA-induced topical inflammation model.

3. 30th International Symposium on the chemistry of Natural Products. Atenas-Grecia. Noviembre 2018. Cape gooseberry (*Physalis peruviana*) modulates the release of inflammatory mediators in LPS-activated RAW 264.7 macrophages.

4. VI Congreso Latinoamericano de Plantas Medicinales. Trujillo-Peru. Agosto 2018. Evaluation of antioxidant activity of six fruit extracts cultivated in Colombia.

5. VI Congreso Latinoamericano de Plantas Medicinales. Trujillo-Peru. Agosto 2018. Anti-inflammatory and antioxidant activity from the fruit of *Annona squamosa* L.

6. XXVI SILAE Congress. Cartagena-Colombia. Septiembre 2017. Anti-inflammatory and anti-oxidant potential of seed extracts of *Ambrosia cumanensis*.

7. XXVI SILAE Congress. Cartagena-Colombia. Septiembre 2017. Antioxidant potential of fruits cultivated in Colombia and identification of phenolic and flavonoids compounds.

8. XXVI SILAE Congress. Cartagena-Colombia. Septiembre 2017. Extracts of *Trichilia hirta* and *Hyptis capitata* exhibited antioxidant and anti-inflammatory activity *in vitro.*

9. V Congreso Latinoamericano de plantas medicinales. La paz-Bolivia. Agosto 2017. Frutas cultivadas en Colombia modulan la liberación de mediadores inflamatorios en macrofagós RAW 264,7.

10. V Congreso Latinoamericano de plantas medicinales. La paz-Bolivia. Agosto 2017. Actividad anti-inflamatoria y antioxidante *in vitro* de extractos de hojas y frutos de *Mammea americana*.

11. V Congreso Latinoamericano de plantas medicinales. La paz-Bolivia. Agosto 2017. Composición fitoquímica y determinación *in vitro* del potencial antioxidante de cinco extractos de frutas tropicales.

12. XXV Silae Congress. Modena-Italia. Septiembre de 2016. Anti-inflammatory

screening of plant extracts used in folk medicine of Colombian Carribean Coast.

13. V Congreso Iberoamericano de Productos Naturales. Bogotá-Colombia. Abril de 2016. Actividad antioxidante y contenido de fenoles totales de los frutos de *Solanum betaceum* y *Solanum quitoense.*

14. III Congreso Latinoamericano de Plantas - Antonio Brack Egg. Iquitos-Perú. Agosto de 2015. Actividad antiinflamatoria de 20 especies de plantas medicinales usadas en la Costa Caribe Colombiana.

Apéndice 3. Premios

1. Premio a la investigación en la modalidad oral del XX Congreso Nacional de Estudiantes de Química Farmacéutica. Evaluación *in vitro* del potencial antioxidante de doce extractos de frutas cultivadas en Colombia, sobre la línea celular macrófagos RAW 264.7 estimulados con LPS. Barranquilla-Colombia. Noviembre 2019.

2. Premio al mejor poster presentado en el 30th International Symposium on the chemistry of Natural Products ISCNP30 & ICOB10). Cape gooseberry (*Physalis peruviana)* modulates the release of inflammatory mediators in LPS-activated RAW 264.7 macrophages. Atenas-Grecia. Noviembre 2018.

3. Premio al mejor poster presentado en el XXVI SILAE Congress. Anti-inflammatory and antioxidant potential of seed extracts of *Ambrosia cumanensis*. Cartagena-Colombia. Septiembre 2017.

4. Premio al segundo mejor trabajo presentado en la convención científica. Categoría salud. Evaluación de la actividad anti-inflamatoria *in vitro* de extractos de plantas de la Costa Caribe Colombiana sobre la línea celular RAW 264.7. Universidad de Cartagena. Octubre 2014.

Apéndice 4. Avales Éticos

Universidad de Cartagena
Fundada en 1827

PRESIDENTE DEL COMITÉ DE ÉTICA EN INVESTIGACIONES DE LA UNIVERSIDAD DE CARTAGENA

HACE CONSTAR

Que, el proyecto titulado "**EVALUACIÓN DEL EFECTO INMUNOMODULADOR RELACIONADO CON EL CONSUMO DE FRUTAS CULTIVADAS EN COLOMBIA, EN UN MODELO DE ENFERMEDAD INFLAMATORIA INTESTINAL INDUCIDA POR DSS**", presentado por **RUBEN DARÍO SALAS DÍAZ**, docente de la Facultad de Ciencias Farmacéuticas de la Universidad de Cartagena, se ajusta a los requerimientos de los referentes éticos contemplados en la resolución 8430 de 1993 del Ministerio de Salud y al Reglamento de Ética de la Universidad de Cartagena, tal como consta en el Acta N° 74 del Comité de Ética en Investigaciones del día 5 de junio de 2014.

Para constancia se firma en la ciudad de Cartagena, a los once (11) días del mes de junio del año dos mil catorce (2014).

ALVARO OLIVERA DIAZ, MD
Presidente

Mayra Martinez

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Universidad de Cartagena
Vice-Rectoría de Investigaciones

EL PRESIDENTE DEL COMITÉ DE ÉTICA EN INVESTIGACIONES DE LA UNIVERSIDAD DE CARTAGENA

CERTIFICA QUE:

El proyecto titulado **ESTUDIO DE LA ACTIVIDAD ANTIINFLAMATORIA Y ANTIOXIDANTE DE TRES ESPECIES DE PLANTAS AMPLIAMENTE UTILIZADAS EN LA MEDICINA POPULAR DE LA COSTA NORTE COLOMBIANA, presentado por el doctor LUIS ALBERTO FRANCO OSPINA,** docente investigador de la Facultad de Ciencias Farmacéuticas de la Universidad de Cartagena, no presenta impedimentos éticos, de acuerdo a lo contemplado en la Resolución 8430 de 1993 del Ministerio de Salud y en el Reglamento de Ética de la Universidad de Cartagena, tal como consta en el Acta N° 32 del Comité de Ética en Investigaciones del 04 de agosto de 2011.

Para constancia se firma en la Ciudad de Cartagena, a los cinco (05) días del mes de agosto del año 2011.

ALVARO OLIVERA DIAZ, MD
Presidente

Mayra Martinez