



**UNIVERSIDAD AUTÓNOMA DEL
ESTADO DE MORELOS**

UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MORELOS
INSTITUTO DE INVESTIGACIÓN EN CIENCIAS BÁSICAS Y APLICADAS
CENTRO DE INVESTIGACIÓN EN DINÁMICA CELULAR

**“EFECTO DE LAS SUBFRACCIONES DE *Cucumis sativus*
SOBRE ADIPOCITOS DISFUNCIONALES”**

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PRESENTA

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PRESENTE

Atendiendo a la solicitud para emitir DICTAMEN sobre la revisión de la TESIS titulada: **“Efecto de las subfracciones de *Cucumis sativus* sobre adipocitos disfuncionales”** que presenta la alumna **Marisol Méndez Martínez (5620120401)** para obtener el título de **Doctor en Ciencias**.

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Resumen

La obesidad es originada por una acumulación excesiva de energía en forma de grasa en el tejido adiposo. Este exceso de energía acumulada induce disfunción en adipocitos, la cual se caracteriza por resistencia a la insulina y por incremento en lipólisis, estrés oxidativo y la secreción de citocinas pro-inflamatorias. El objetivo de este trabajo es evaluar el efecto de las subfracciones de *Cucumis sativus* sobre la resistencia a la insulina en adipocitos disfuncionales *in vitro* e *in vivo*. Para satisfacer dicho objetivo, se indujo disfunción a los adipocitos con dexametasona o IL-1 β , posteriormente fueron incubados con diferentes concentraciones de las tres subfracciones (SF1, SF2 o SF3) de *Cucumis sativus* durante 24 horas. Se evaluó la resistencia a la insulina, a través del consumo de 2-desoxiglucosa y la liberación de glicerol. Con base en estos resultados se construyó el modelo de superficie, lo que permitió determinar 5 posibles mezclas de las subfracciones (M1, M2, M3, M4 y M5). Se indujeron adipocitos disfuncionales con 1 ng/ml de IL-1 β y se evaluó el efecto de las 5 subfracciones en la resistencia a la insulina aunada al estrés oxidante y al estado proinflamatorio. Las mezclas M4 (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 30 μ g/mL) y M5 (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 10 μ g/mL) controlaron con mayor eficiencia los parámetros evaluados de manera satisfactoria dichos parámetros

Por otro lado, a ratones C57BL/6J se les indujo obesidad mediante la ingesta crónica de una dieta hipercalórica durante 15 semanas, y se trataron por 10 semanas más con la combinación M5 vía oral. Los parámetros metabólicos (triglicéridos, colesterol, curvas de tolerancia a la glucosa, a la insulina y a la glucosa con insulina), físicos (peso e índice de Lee), fueron evaluados en la semana 0, 15 y 25. La concentración de citocinas (IL-6, IL-10 y TGF β) se evaluó a la semana 25 en tejido adiposo subcutáneo e hígado.

Los resultados *in vitro* indicaron que la SF1 (40 μ g/mL) y SF2 (40 μ g/mL) y SF3 (20 μ g/mL) inducen un consumo de 2-desoxiglucosa del 87%, 57% y 87%, respectivamente. Por otro lado, SF1 (5 μ g/mL) y SF2 (5 μ g/ml), y SF3 (40 μ g/mL) incrementan la secreción de glicerol un 10.6%, 18.9% y 11.8%, respectivamente. El modelo de superficie mostró 5 combinaciones efectivas, de las cuales M4 (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 30 μ g/mL) y M5 (SF1 40 μ g/mL: SF2 60 μ g/mL:SF3 10 μ g/mL) fueron capaces de controlar la resistencia a la insulina, el estado pro-oxidante y el estado pro-inflamatorio evaluadas en adipocitos 3T3-L1 disfuncionales. Los compuestos presentes en las subfracciones son principalmente glicina, asparagina y arginina.

En ratones obesos, la combinación M5 no modificó 4 parámetros: el peso, índice de Lee, triglicéridos ni colesterol; pero si fue eficiente para el control de la intolerancia a la glucosa y la resistencia a la insulina, así como también indujo un ambiente inmunológico regulador en tejido adiposo subcutáneo e hígado al disminuir la concentración de IL-6 y aumentar la concentración de IL-10 y TGF- β . En conclusión, las subfracciones SF1, SF2 y SF3 de la fracción acuosa de *Cucumis sativus* y sus combinaciones M4 y M5 son

efectivas para el control de adipocitos disfuncionales y la combinación M5 para el control de la intolerancia a la glucosa, la resistencia a la insulina y el estado proinflamatorio en ratones obesos.

Abstract

Obesity is caused by an excessive storage of energy as fat in adipose tissue. This excess of stored energy induces adipocyte dysfunction, characterized by insulin resistance and by an increase in lipolysis, oxidant stress, and the secretion of proinflammatory cytokines. This work is aimed to evaluate the effect of various *Cucumis sativus* subfractions on insulin resistance in dysfunctional adipocytes *in vivo* and *in vivo*. To satisfy this objective, dysfunction was induced to adipocytes with dexamethasone or IL-1 β , subsequently they were incubated with differing concentrations of the three *C. sativus* subfractions assayed (SF1, SF2, or SF3) for 24 h. Insulin resistance was evaluated, through the consumption of 2-deoxyglucose and the release of glycerol. Based on these results, the surface model was constructed, which allowed determining 5 possible mixtures of the subfractions (M1, M2, M3, M4 and M5). Dysfunctional adipocytes were induced with 1 ng/ml of IL-1 β and the effect of the 5 subfractions on insulin resistance combined with oxidative stress and proinflammatory state was evaluated. The M4 mixtures (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 30 μ g/mL) and M5 (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 10 μ g/mL) controlled the evaluated parameters with greater efficiency satisfactorily said parameters

On the other hand, C57BL/6J mice were induced obesity by chronic intake of a hypercaloric diet for 15 weeks, and were treated for 10 more weeks with the M5 combination orally. The metabolic parameters (triglycerides, cholesterol, glucose tolerance curves, insulin and glucose with insulin), physicals (weight and Lee index), were evaluated at week 0, 15 and 25. The concentration of cytokines (IL-6, IL-10 and TGF β) was evaluated at week 25 in subcutaneous adipose tissue and liver.

The *in vitro* results indicated that that SF1 (40 μ g/mL) and SF2 (40 μ g/mL) and SF3 (20 μ g/mL) induce a 2-deoxyglucose consumption of 87%, 57% and 87%, respectively. On the other hand, SF1 (5 μ g/mL) and SF2 (5 μ g/mL), and SF3 (40 μ g/mL) increase the secretion of glycerol by 10.6%, 18.9%, and 11.8%, respectively. The surface model showed 5 effective combinations, of which M4 (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 30 μ g/mL) and M5 (SF1 40 μ g/mL: SF2 60 μ g/mL: SF3 10 μ g/mL) were able to control insulin resistance, the pro-oxidant state and the pro-inflammatory state evaluated in

dysfunctional 3T3-L1 adipocytes. The compounds present in the subfractions are mainly glycine, asparagine and arginine.

In obese mice, the M5 combination did not modify 4 parameters: weight, Lee index, triglycerides or cholesterol; but it was efficient for the control of glucose intolerance and insulin resistance, as well as it induced a regulatory immunological environment in subcutaneous adipose tissue and liver by decreasing the concentration of IL-6 and increasing the concentration of IL-10 and TGF- β . In conclusion, the subfractions SF1, SF2 and SF3 of the aqueous fraction of *Cucumis sativus* and its combinations M4 and M5 are effective for the control of dysfunctional adipocytes and the combination M5 for the control of glucose intolerance, insulin resistance and the proinflammatory state in obese mice.

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ABREVIATURAS

ADN: Ácido Desoxirribonucleico
ADD1: Factor 1 Dependiente de Diferenciación de Determinación de Adipocitos
AE: Acetoetílico
AGEs: Productos Finales de la Glicación Avanzada
ALEs: Productos de la Lipoperoxidación Avanzada
Aq: Acuoso
C/EBP: Proteína de Unión a CCAAT
CHOP: Proteína Homóloga C/EBP
Cs: *Cucumis sativus*
DHC: Dieta Hipercalórica
DM: Diabetes mellitus
Glut 4: Transportador de glucosa 4
HA: Hidroalcohólico
HDL: Lipoproteínas de Alta Densidad
HNE: 4-hidroxi-trans-2-nonenal
IL: interleucina
IMC: índice de Lee
IRS: Sustratos del Receptor de Insulina
LDL: Lipoproteínas de Baja Densidad
LDLox: Lipoproteínas de Baja Densidad Oxidadas
MCP-1: Proteína Quimioatrayente Monocítica
MDA: Malondialdehido
NO: Óxido Nítrico
O₂⁻: Anión superóxido
PAI-1: Inhibidor del Plasminógeno Tisular
PCR: Proteína C Reactiva
PPAR: Receptor Activador de Proliferación de Peroxisomas
RE: Retículo Endoplásmico
ROS: Especies Reactivas de Oxígeno
SM: Síndrome Metabólico
TAB: Tejido Adiposo Blanco
TAS: Tejido Adiposo Visceral
TAV: Tejido Adiposo Subcutáneo
TGF- β : Factor de Crecimiento Transformante
TNF- α : Factor de Necrosis Tumoral alfa
Treg: Células T reguladoras
URP: Proteínas Mal Plegadas
VLDL: Lipoproteína de Muy Baja Densidad

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1. Introducción

1.1. Tejido Adiposo y su función

El tejido adiposo blanco (TAB) juega un papel crítico en el almacenamiento y homeostasis de energía. Dicha energía se almacena en forma de triglicéridos y es liberada en forma de ácidos grasos no esterificados en casos de inanición (Arner and Langin 2014). Además de los adipocitos, el TAB, también presenta otros tipos celulares como son células endoteliales, pericitos y macrófagos (Vidal *et al.*, 2008). Este tejido se diferencia en tejido adiposo visceral (TAV) y tejido adiposo subcutáneo (TAS). Siendo el primero, más notable en individuos obesos ya que es el principal depósito de energía (Palomer 2005; Cachofeiro *et al.*, 2006), y se caracteriza porque sus adipocitos son de mayor tamaño, más vascularizados y con gran número de receptores β adrenérgicos por lo que presentan una mayor actividad metabólica (Vega 2010). Entre las que se encuentran: 1) Lipogénesis: que es el almacenaje y síntesis de ácidos grasos; y 2) lipólisis: que es la movilización o hidrólisis de triglicéridos (Sánchez *et al.*, 2005).

Por otro lado, en años recientes se ha observado que el TAV secreta varias moléculas llamadas adipocinas y se encuentra regulado por diferentes estímulos hormonales, por ello se ha definido como un órgano endocrino dinámico (Sánchez *et al.*, 2005; Vega 2011). Estas adipocinas median múltiples procesos incluyendo sensibilidad a la insulina, estrés oxidativo, homeostasis del metabolismo energético, coagulación sanguínea y respuesta inflamatoria (Kaur 2014).

1.2 Adipocitos disfuncionales y obesidad

Los adipocitos, además de liberar ácidos grasos, otros lípidos y metabolitos, secretan más de 600 factores bioactivos conocidos como adipocinas (Bastard *et al.*, 2006), las cuales modulan no solo la adipogénesis, el metabolismo y la función de los adipocitos sino también el apetito y saciedad, distribución del tejido adiposo, secreción y sensibilidad a la insulina, liberación de energía, inflamación, presión sanguínea, homeostasis y función endotelial (Ahima 2006). Por lo que, actúan no solo a nivel del

tejido adiposo sino también a nivel de cerebro, hígado, músculo, endotelio, corazón y células β pancreáticas (Yao *et al.*, 2014). Este patrón de secreción refleja la función de los adipocitos, ya que un desequilibrio en la secreción de adipocinas se relaciona con el desarrollo de patologías, entre ellas la obesidad (Blüher 2014).

La obesidad es un padecimiento crónico, complejo y de etiología multifactorial, producto de un desequilibrio entre la energía ingerida y la energía gastada. El exceso de dicha energía se convierte en grasa y se acumula en los adipocitos, haciendo que estos aumenten de tamaño (hipertrofia), así como en número (hiperplasia) (Cachofeiro *et al.*, 2006, Blancas 2010, De Ferranti 2009). Por otro lado, la prevalencia de la obesidad en países industrializados ha aumentado el doble en estas últimas tres décadas y es considerada como una pandemia. La obesidad se relaciona con la reducción de la calidad de vida, aumentando el riesgo prematuro de muerte y la generación de enfermedades crónicas, incluyendo enfermedad coronaria, hipertensión, diabetes tipo 2 y ciertos tipos de cáncer (Freedman 2011; Fernández *et al.*, 2011). De acuerdo con la Encuesta Nacional de Salud y Nutrición 2012 la prevalencia de la obesidad en México aumentó del 2006 al 2012 un 14.3%, lo que indica que 7 de cada 10 mexicanos adultos son obesos. Debido a esta incidencia se ha buscado combatir la obesidad con base en una dieta baja en calorías, evitar el sedentarismo y generar estrategias farmacológicas que modulen la obesidad así como sus patologías asociadas (Gutiérrez *et al.*, 2012).

1.2.1 Metainflamación

Como ya se mencionó anteriormente, el tejido adiposo además de su función como reservorio de lípidos, también es conocido como un órgano activo endocrino que produce y secreta una variedad de adipocinas (Bastard *et al.*, 2006; Manabe 2011). En condiciones de obesidad, su secreción normal se ve alterada por lo que su homeostasia se ve interrumpida. Este evento estimula múltiples cascadas de señalización, entre ellas la de NF- κ B, que llevan finalmente a la producción de diferentes marcadores biológicos de inflamación (Bastard *et al.*, 2006) tales como proteína C reactiva (PCR), TNF- α , interleucina (IL) 6, IL-1 β , Leptina, Angiotensina II, inhibidor del plasminógeno tisular (PAI-1), P-selectina o VCAM-1 (Cachofeiro *et al.*, 2006). Así como también una liberación

exacerbada de ácidos grasos libres debido a la activación de la lipólisis (Manabe 2011). Por otro lado, la secreción de adiponectina disminuye conforme aumenta la masa grasa, este efecto perjudica el almacenaje de triglicéridos e incrementa la liberación de ácidos grasos libres (Bastard *et al.*, 2006). Por lo que, el aumento del consumo de ácidos grasos, que induce hipertrofia en los adipocitos y una alteración en la secreción de adipocinas, genera una respuesta inflamatoria mediada tanto por células del sistema inmune innato (macrófagos) como adaptativo (linfocitos T). Esta respuesta se denomina inflamación crónica de bajo grado asociada a la obesidad, también conocida como metainflamación (Bastard *et al.*, 2006; Vidal *et al.*, 2008; Yao *et al.*, 2014). En condiciones normales la homeostasis del tejido adiposo está mantenida por la presencia de IL-10 y TGF- β , las cuales son secretadas por las células T reguladoras (Treg) y macrófagos tipo II con perfil antiinflamatorio. Sin embargo en condiciones de obesidad aumenta la secreción de IL-6 y TNF α , potenciando el proceso de metainflamación (Yao *et al.*, 2014). En años recientes se ha visto que en obesidad la presencia de TGF- β , IL-17 e IL-6 induce una respuesta inmune inflamatoria Th17 (Chen *et al.*, 2014).

La leptina, una adipocina, es producida principalmente por el tejido adiposo. Su función es la de regular la ingesta de alimento así como el gasto energético y sus niveles están relacionados directamente con el tamaño del adipocito (Abella *et al.*, 2014). En condiciones de obesidad aumenta su producción, sin embargo se presenta un proceso de “resistencia a la leptina”, por lo que no se puede controlar el apetito ni mantener el índice de masa corporal (Ahima 2006). Además, los altos niveles de leptina potencian la metainflamación, ya que es capaz de activar macrófagos e inducir la secreción de TNF- α (Bastard *et al.*, 2006).

Otras adipocinas con propiedades proinflamatorias que se encuentran aumentadas en obesidad son TNF- α , IL-1 β e IL-6. Estas, de manera metabólica generan resistencia a la insulina, debido a que inducen la fosforilación de residuos de serina y treonina; y no de tirosina como es normalmente en los Sustratos del Receptor de Insulina (IRS), impidiendo así la translocación del transportador de glucosa 4 (GLUT 4) (Arner y Lagin 2014). Otro efecto metabólico ocasionado por estas adipocinas, es la inducción de la secreción en el hígado de Lipoproteína de muy baja densidad (VLDL) y triglicéridos por el hígado (Yudkin *et al.*, 2000; Maachi *et al.*, 2004). Por otro lado, la secreción de estas

adipocinas induce la infiltración de macrófagos tipo I que secretan IL-1 β , que, aunado a la secreción de leptina, PCR y resistina por el adipocito potencian la producción de TNF- α e IL-6 y con ello la metainflamación (Figura 1) (Manabe 2011; Harford *et al.*, 2011).

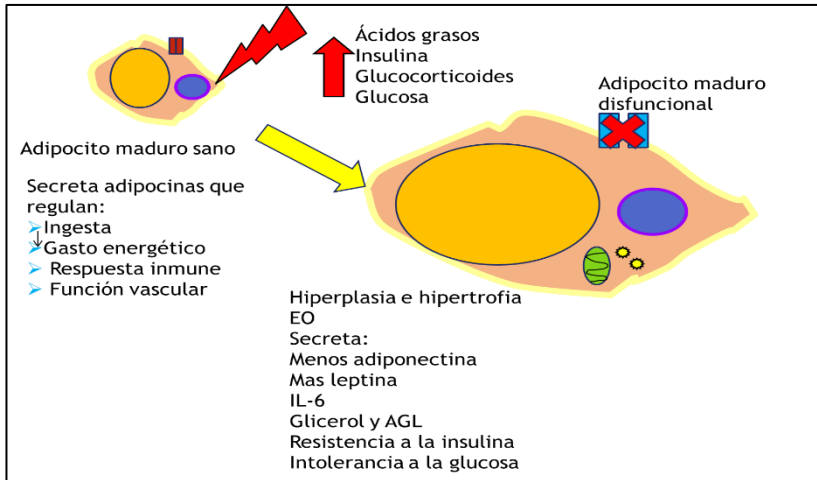


Figura 1. Características y diferencias entre un adipocito maduro sano y un adipocito maduro disfuncional

En estudios recientes se ha observado que existe una relación entre los Lipopolisacáridos (LPS) provenientes de la dieta y el desarrollo de la metainflamación, la cual estaría promoviendo las patologías asociadas a la obesidad (Hersoug *et al.*, 2018). Los LPS se unen a los TLR4 presentes en la superficie de los adipocitos y estos en respuesta inducen la secreción de IL-6 (Ying *et al.*, 2000).

1.2.2 Estrés metabólico y oxidante

En condiciones de obesidad, el incremento de ácidos grasos libres, la hiperglucemia y la hiperinsulinemia, están relacionados con la disfunción en los adipocitos, principalmente el estrés metabólico en retículo endoplasmático (RE) y en mitocondria (estrés oxidante) (Chen 2006). El primero se refiere al mal funcionamiento del RE, por lo que el correcto plegamiento y modificación de proteínas, síntesis lipídica y almacenamiento de Ca²⁺ están inhibidos. Se han descrito dos manifestaciones del estrés del RE: 1) el incremento de lactato y la producción de la Proteína Homóloga C/EBP (CHOP), lo que disminuye la producción de adiponectina; y 2) la respuesta de proteínas mal plegadas (URP, por sus siglas en inglés Unfolded Protein Response), las cuales se agregan en el citosol interfiriendo con las funciones de la célula. Ambas manifestaciones

pueden aumentar la secreción de lípidos y adipocinas potenciando la metainflamación (Gregor y Hotamisligil 2007; Hosogai et al., 2007). En cuanto al estrés de la mitocondria u oxidativo se da por un desequilibrio en los niveles de especies reactivas de oxígeno (ROS) vs moléculas antioxidantes durante el procesamiento del exceso de ácidos grasos libres, el cual produce un desacoplamiento en la cadena respiratoria. Los niveles elevados de ROS, por ejemplo, el anión superóxido (O_2^-), promueven la oxidación de glucosa y lípidos, generando Productos Finales de la Glicación Avanzada (AGEs) y Productos de la Lipoperoxidación Avanzada (ALEs) respectivamente (De Ferranti et al 2009; Murdolo et al., 2013, Ott et al., 2014).

Por otro lado, en adipocitos cultivados con diferentes concentraciones de corticoesteroides o citosinas proinflamatorias, se aumenta la liberación de ácidos grasos libres, glicerol y citosinas proinflamatorias, los cuales interactúan con los complejos I y II de la cadena respiratoria lo que induce un incremento en la concentración de O_2^- , potenciando el estado disfuncional del adipocito (Martyn *et al.*, 2008; He et al, 2017; Schönfeld et al., 2008).

1.2.3 Intolerancia a la glucosa y resistencia a la insulina

El aumento de lípidos y de estrés oxidativo observado en la obesidad da como resultado consecuencias tanto intracelulares (locales) como sistémicas. Las primeras se refieren a la producción y secreción exacerbada de adipocinas y ácidos grasos libres, así como la intolerancia a la glucosa y la resistencia a la insulina en adipocitos (De Ferranti 2009). Entre las segundas figuran la resistencia a la insulina, la intolerancia a la glucosa, la dislipidemia y la disfunción endotelial (Manabe 2011; Bastard *et al.*, 2006; Wajchenberg *et al.*, 2009).

La glucosa es el principal combustible usado en humanos y es la única fuente de energía para el cerebro. Su homeostasis está controlada por la insulina (Abdul *et al.*, 2006), una hormona sintetizada por las células β del páncreas, que es secretada a la sangre y ejerce su acción por unión a su receptor. Una vez activado el receptor, se generan “segundos mensajeros” que estimulan dos vías de acción: I) La vía de PI3K (cascada de reacciones de fosforilación por una cinasa), que favorece el transporte de

glucosa al interior de la célula; y II) la vía MAPK que promueve el crecimiento, la diferenciación y proliferación celular (Menéndez *et al*, 2009). La insulina es una hormona pleiotrópica ya que no solo estimula la captación de la glucosa por el músculo, hígado y tejido adiposo, si no también activa la síntesis de glucógeno en hígado y músculo; así como la deposición de lípidos en tejido adiposo (Jeevendra *et al.*, 2008).

Sin embargo, cuando la secreción de insulina y su acción se deterioran, las células β del páncreas se tornan incapaces de sostener el estado de hiperinsulinemia lo que induce una hiperglucemia crónica, el cual es un rasgo característico para el desarrollo de Diabetes Mellitus tipo 2 (DM2) (Proença *et al.*, 2014). Otro efecto es la resistencia a la insulina, la cual es definida como la disminución de la capacidad de la insulina para ejercer sus acciones biológicas en tejidos diana típicos, como son el músculo esquelético, el hígado o el tejido adiposo. Estos eventos resultan en intolerancia a la glucosa con alteración de la vía de PI3K, en el que los residuos de serina y treonina se fosforilan en lugar de los residuos de tirosina habituales en los IRS, inhibiendo la expresión de GLUT 4 en la superficie de la célula y con ello el transporte y la utilización de glucosa. Por otro lado, la hiperinsulinemia sobre estimula la vía MAPK que potencia la acción mitogénica y la migración de las células musculares lisas de los vasos, favoreciendo la aterogénesis (Menéndez *et al*, 2009) (Figura 2). Como se mencionó anteriormente la presencia de adipocinas y estrés metabólico disminuye la sensibilidad a la insulina (Arner y Lagin 2014). Es por estos eventos que las personas que presentan obesidad tienden a desarrollar DM2 (Gideon *et al.*, 2008).

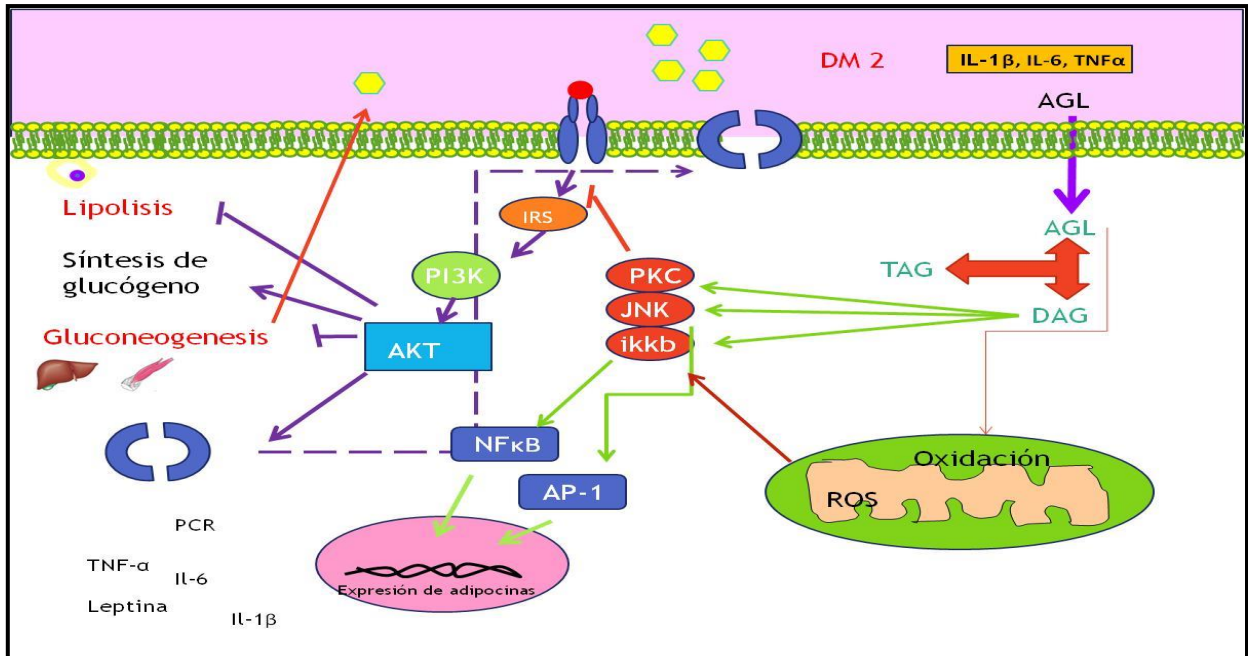


Figura 2. Resistencia a la insulina e inflamación. La insulina se une a la subunidad α del receptor de insulina, y estimula la autofosforilación de la subunidad β y la activación del receptor. Con ello se fosforilan los IRS. Los cuales estimulan otras cascadas de señalización, entre las que se encuentra la catalizada por la enzima PI₃ cinasa la cual media la síntesis de glucógeno; inhibición de la lipólisis y gluconeogénesis; la translocación de GLUT4 a la superficie de la célula y con ello la internalización de glucosa. Al haber un incremento de ácidos grasos libres se estimula la generación de ROS que activan otras vías de señalización como la PKC la cual inhibe la autofosforilación de los IRS impidiendo de esa forma la expresión de GLUT4 incrementando las concentraciones de glucosa libre. La vía de NF κ B la cual regula la expresión de citoquinas proinflamatorias. Potenciando el proceso inflamatorio y la RI.

1.2.4 Dislipidemia

La dislipidemia se caracteriza fundamentalmente por el aumento de triglicéridos y de lipoproteínas de baja densidad (LDL), así como una disminución de lipoproteínas de alta densidad (HDL) (Proença *et al.*, 2014). Las lipoproteínas son partículas formadas por una fracción proteica llamada apoproteína y una fracción lipídica, su función es la de solubilizar y transportar lípidos en el plasma. El aumento de las LDL caracteriza al fenotipo aterogénico. Entre las características bioquímicas que incrementan este fenotipo, se señala el menor contenido en fosfolípidos y colesterol no esterificado en su superficie, el cual induce cambios en la conformación de la apolipoproteína B-100 lo que conduce a la mayor afinidad de estas partículas por proteoglicanos arteriales; también se señala su mayor facilidad para el transporte dentro del espacio subendotelial y mayor

susceptibilidad a la oxidación lipídica (Gideon *et al.*, 2008). Cuando la capacidad del tejido adiposo es sobrepasada, la conversión a VLDL o partículas semejantes se retrasa y se da la hipertrigliceridemia, por lo que el almacenamiento de grasa se vuelve ectópico dañando hígado, músculo, páncreas y corazón (Laclaustra *et al.*, 2007).

1.3 Tratamientos farmacológicos

La obesidad es un problema a nivel mundial y el tratamiento convencional consiste en la administración crónica de medicamentos que actúen venciendo la resistencia a la insulina, disminuyendo la presión arterial así como los niveles séricos de lípidos y glucosa (Nuernberg *et al.*, 2011). Debido a que aún no se cuenta con un medicamento que controle la obesidad y sus patologías asociadas, es necesario que el paciente sea tratado a través de una polifarmacia que puede causar efectos secundarios, además de ser costosos para el paciente y el país (Hwang *et al.*, 2011).

Los tratamientos que se prescriben normalmente son: un antihiper glucémico como la metformina que actúa disminuyendo la gluconeogénesis en el hígado y permitiendo la captura de glucosa por la célula muscular. Sin embargo el uso crónico de este medicamento puede inducir insuficiencia renal, hepática y cardíaca, así como acidosis metabólica aguda o crónica (Mata 2008); y un anti-dislipidémico como el fenofibrato el cual es un agonista del PPAR α por lo que disminuye la concentración de triglicéridos, sin embargo el consumo de este medicamento puede provocar molestias gastrointestinales, náuseas, debilidad muscular (Uchida *et al.*, 2010). Por ello es importante generar estrategias farmacológicas integrales que controlen la obesidad y sus patologías asociadas.

1.4 Flavonoides, polisacáridos y aminoácidos para el control de la disfunción en adipocitos

El uso de extractos de plantas en la medicina tradicional para el tratamiento de una amplia gama de trastornos en la salud ha ido en aumento. Y debido a los grandes beneficios que se han observado en el control de las patologías asociadas a la disfunción

de adipocitos, es que se ha desarrollado la búsqueda de los compuestos responsables de esos beneficios, por lo que se han encontrado que los polisacáridos, flavonoides y aminoácidos, son los responsables de conferir esas propiedades.

Por ejemplo, se ha visto que los flavonoides provenientes de *Crataegus pinnatifida* disminuyen el colesterol y la concentración sérica de triglicéridos; además de disminuir la actividad de la lipasa lipoproteica en tejido adiposo con lo que se disminuye la acumulación de grasa (Liang *et al.*, 2010). Chen y colaboradores (2010) observaron que el resveratrol tiene la capacidad de inhibir la adipogénesis al actuar directamente sobre C/EBP α y PPAR γ . Los flavonoides y taninos provenientes del fruto de *Cucumis sativus* pueden actuar como “scavenger” de radicales libres, por lo que son considerados como antioxidantes y antiinflamatorios (Kumar D. *et al.*, 2010; Singh N. *et al.*, 2010). En cuanto a los polisacáridos, se ha observado que participan en la regulación de la respuesta inmune, ya que tres polisacáridos llamados lentinan, schizophyllan y polisacárido unido a proteína (PSK), aislados de *Lentinan edodes*, *Schizophyllum commune* y *Coriolus versicolor*, respectivamente, inducen la secreción de citocinas inmunomoduladoras, como IL-10, así como sus receptores (Ooi V.E. y Liu F. 2010). Otra propiedad de los polisacáridos que se ha observado, es la de reducir las concentraciones del colesterol y triglicéridos plasmáticos; y por otro lado aumenta las concentraciones de HDL (Zhong *et al.*, 2010; Ning N *et al.*, 2013) indicando que, estos compuestos, juegan una papel importante en la regulación tanto el estado inmunológico como el estado metabólico. Por otro lado, los aminoácidos, además de su papel como constituyentes de proteínas y polipéptidos, son reguladores importantes en las vías necesarias para el mantenimiento, crecimiento y reproducción celular, así como para el metabolismo sistémico y la inmunidad, favoreciendo el procesamiento de nutrientes y la acumulación de proteínas, disminuyendo la adiposidad y mejorando la salud. Por otro lado, se ha observado que el aminoácido S-metil cisteína sulfóxido (SMCS), proveniente de *Allium cepa*, no solo controla la concentración de glucosa y lípidos en suero y tejidos, si no también normaliza la actividad de la hexoquinasa hepática, glucosa-6-fosfatasa y de la HMGCoA reductasa (Kumari K *et al.*, 1995). Y aminoácidos provenientes de *Panax ginseng*, han demostrado su actividad como “scavenger” de radicales libres (Eun *et al.*, 2008), controlando de esa manera el estado oxidante que se presenta en condiciones de obesidad (Chen 2006).

1.5 Modelo de superficie de respuesta

El modelo de superficie de respuesta es un conjunto de técnicas matemáticas que permite analizar un efecto que está influenciado por varias variables de carácter cuantitativo. Su objetivo es encontrar los valores óptimos de las variables independientes para encontrar la variable dependiente o de respuesta que se busca. Para ello, es necesario que la variable de respuesta que se desea analizar se encuentre adecuadamente establecida (Zambrano *et al.*, 2014).

En la fase inicial del estudio se trata de identificar la región de respuesta óptima y para ello se utilizan parámetros factoriales completos 2^k o fraccionarios 2^{k-p} , para cada factor se consideran tres niveles, que pueden codificarse en los valores +1(más alto), 0 (intermedio) y -1 (más bajo). Considerando todas las posibles combinaciones de los niveles de los K factores, se obtiene una matriz de diseño de 2^k filas, cada una de las cuales representa un tratamiento. Dado que el diseño de experimentación consta de tres niveles en cada factor, se escribe un modelo de segundo orden dado por la siguiente ecuación:

$$Y_n = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j=1}^3 b_{ij} X_i X_j$$

Donde Y_n es la respuesta de las variables, b_0 es el coeficiente de regresión y X_i es el nivel codificado de cada variable independiente.

En la figura 3 se observa la representación gráfica del modelo de superficie.

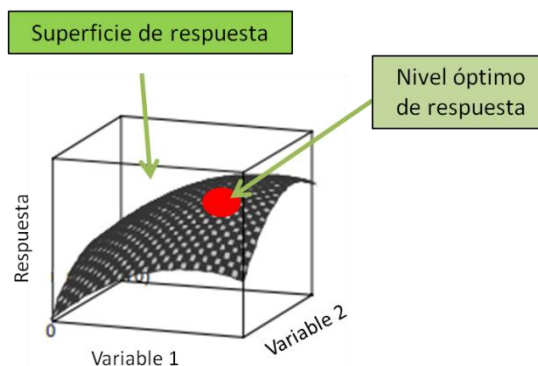


Figura 3. Representación gráfica del modelo de superficie (Alfaro Villanueva J. 2014)

2. Antecedentes

2.1 Taxonomía y botánica de *Cucumis sativus* (pepino)

Planta herbácea anual, recubierta de pelos erizados, de raíces fasciculadas y desarrollo bastante superficial. Tallos trepadores o rastreros muy ramificados en la base, con cuatro ángulos marcados y zarcillos simples. Sus hojas son largamente pecioladas, fuertemente cordadas en la base, con el ápice acuminado, en cuyo limbo se aprecian de 3 a 5 lóbulos. Sus Flores son unisexuales, de localización axilar y color amarillento; inicialmente se forman las flores masculinas y posteriormente las femeninas (Maroto 2002). Los frutos son pepónides de tamaño y forma variables. El color de su corteza puede ser verde, amarillo o blanco, mientras que la pulpa es siempre blanca y acuosa. Las semillas son alargadas, ovas, aplastadas y de color amarillento. La viabilidad de las semillas dependerá de las condiciones ambientales y del origen de la semilla, que puede ser desde unas semanas hasta 5 años (Maroto 2002; Monroy y Monroy 2006).

Pertenece a la familia de las Cucurbitaceae y su nombre científico es el de *Cucumis sativus* (Pepino) (Maroto 2002).

Información taxonómica

REINO : *Plantae*

DIVISIÓN : *Magnoliophyta*

CLASE : *Magnoliopsida*

ORDEN : *Violales*

FAMILIA : *Cucurbitaceae*

GÉNERO : *Cucumis* L., 1753

ESPECIE : *Cucumis sativus* L., 1753

2.2 Etnofarmacología de *Cucumis sativus*

Cucumis sativus fue introducida al país después de la conquista, motivo por el cual no se tiene registrado su uso etnofarmacológico en la Medicina Tradicional Mexicana.

Sin embargo, en las revistas indizadas se tienen registros formales del uso de plantas de la familia de las Cucurbitaceae a la que pertenece el pepino, así como del género *Cucumis* y solo algunos para la especie *sativus* para el control en forma aislada de la inflamación, del estrés oxidante, de la hiperglucemia, diabetes, dislipidemia y de la hipertensión (Ghule *et al.*, 2009; Naik *et al.*, 1980; Naito *et al.*, 2005; Veeramani *et al.*, 2010; Vouldoukis *et al.*, 2004; Zuhair *et al.*, 2000).

Algunos de los compuestos activos presentes en *Cucumis sativus* que podrían estar participando en algunas de las propiedades antes mencionadas han sido descritos. Estos son: a) Cucurbitacinas: miembro de los triterpenos, caracterizadas por dar el sabor amargo y su toxicidad, la conforman 12 miembros de los cuales *Cucumis sativus* tiene la A, B, C, D, E e I.(Rice *et al.*, 1981); b) C-glucosidos flavonas: Cucumerina A y B, Vitexina, Isovitexina, Orientina, Isoorientina, saponarina, ácido 4-hidroxicinnámico (Aboud-Zaid *et al.*, 2001); c) Megastigmane: I y II(Kai *et al.*, 2007); d) Flavonoides: Kaempferol, quercetina, Isoramnetina(Krauze-Baranowska y Cisowski W. 2001); e) Compuestos fenólicos: p-coumarico, caffeic, ácido ferulico; f) Lutein; (+)-(1R,2S,5R,6S)-2,6-di-(4'-hydroxyphenyl)-3,7-dioxabicyclo (3,3,0) octane; indole-3-aldehyde; indole-3-carboxylic acid y adenosina(Kai *et al.*, 2007).

2.2.1 Los extractos: acuoso y acetoetilico de *Cucumis sativus*, controlan la inflamación y las patologías asociadas a la obesidad

En estudios recientes se observó que la fracción acuosa de la fruta de *Cucumis sativus* (*Aq-Cs*) puede actuar como antioxidante y antiinflamatorio (Kumar *et al.*, 2010; Naresh *et al.*, 2010) gracias a la presencia de flavonoides y taninos, los cuales indican que pueden actuar como "scavenger" de radicales libres. Por otro lado, Mohsen Minalyan y colaboradores (2011) demostraron su efecto antihiperglucemiante de los extractos hidroalcohólico y etanólico de las semillas de *Aq-Cs* en ratas diabéticas inducidas por la administración de Estreptozotocina. Mientras que Mercy y colaboradores (2008) observaron que el aceite de la *Aq-Cs* reduce los niveles de LDL disminuyendo la tasa aterogénica. Este efecto ya había sido observado por Roma-Ramos y colaboradores

(1995). Estos resultados indican que la *Aq-Cs* tiene propiedades que pueden ser utilizadas para controlar al síndrome metabólico.

En un trabajo previo de nuestro grupo de investigación, fue evaluado el efecto del extracto hidroalcohólico (HA-Cs), y las fracciones acetoetilica (AE-Cs) y *Aq-Cs* sobre el estado de metainflamación en la obesidad y sus patologías asociadas. Para ello se utilizó un modelo murino inducido por la ingesta crónica de una dieta hipercalórica (DHC) durante 12 semanas, elevando peso, densidad, glucosa, IL-6, IL-10 e IL-1 β , posteriormente se administraron vía oral el HA-Cs, y las AE-Cs y *Aq-Cs*, de manera individual, durante 10 semanas, confirmando que la *Aq-Cs* y *HA-Cs* normalizaron los niveles de glucosa, IL-6, IL-10 e IL-1 β . Este estudio señala el interés de ambos extractos para normalizar el estado glicémico e inflamatorio asociado a la obesidad (Arteaga *et al.*, 2011).

2.2.2 La fracción acuosa de *Cucumis sativus* induce un ambiente inmunoregulador tanto *in vivo* como *in vitro*

Con la intención de conocer la base de los resultados anteriores y si se relacionaban con la regulación de la respuesta inmune en obesidad, se evaluó la capacidad del *Aq-Cs* para inducir un ambiente inmunoregulador a través de la expresión de IL-10 y TGF- β en tejidos adiposos y riñón. Además de la capacidad de inducir células T reguladoras Foxp3⁺/CD4⁺/CD25⁺ (Treg) en condiciones inductoras de TH17. Ya que se ha reportado que las Treg son una población celular que controla la respuesta inmune, que también se han visto involucradas en controlar el ambiente originado por la obesidad y la metainflamación (Cipolletta *et al.*, 2011). Para ello, se utilizó el mismo modelo murino de obesidad y se cuantificó la secreción de citocinas reguladoras antes mencionadas. Los resultados obtenidos indicaron que los compuestos presentes en el *Aq-Cs*, posiblemente controlaron la meta-inflamación y las alteraciones metabólicas presentes en ratones obesos a través de la inducción de un ambiente regulador. Después de 10 semanas de tratamiento, el *Aq-Cs* disminuyó la acumulación de TAVE y TAS, venció la intolerancia a la glucosa y la resistencia a la insulina e indujo un ambiente regulador a través de la expresión de IL-10 y TGF- β en los tejidos adiposos. La capacidad de los extractos de

generar Treg se comprobó *in vitro* ya que indujo estas células en condiciones inductoras de TH17 a manera dosis dependiente (Méndez *et al.*, 2014).

3. Justificación

Debido a los malos hábitos que se han adquirido en los recientes años, tales como sedentarismo, ingesta de alimentos altos en calorías, entre otros, ha aumentado el riesgo de padecer sobrepeso y generar obesidad. Esta enfermedad es un problema de salud grave y creciente que se asocia a un estado proinflamatorio crónico de bajo grado que juega un papel muy importante en la generación de patologías como resistencia a la insulina, intolerancia a la glucosa, dislipidemia y disfunción endotelial que a su vez llevan a DM2 y a enfermedades cardiovasculares, que en conjunto se conoce como SM. México ha sido ubicado por la Encuesta Nacional de Salud y Nutrición 2012 como el primer lugar de prevalencia mundial en obesidad, lo que indica que se deben generar estrategias farmacológicas alternas para el control de esta patología. Sin embargo, a la fecha no existe un fármaco que sea capaz de controlar de manera integral al SM, por lo que debe ser controlado de manera individual. Esta polifarmacia genera, por un lado gastos económicos muy altos tanto a los pacientes como al país y por otro lado efectos secundarios propios del tratamiento ingerido. Una alternativa es el uso de fitomedicamentos que según la ley general de Salud de 1998 los define como un extracto vegetal conformado por una mezcla de compuestos que pueden modificar la evolución y las complicaciones de una enfermedad, por lo tanto, son una alternativa real, para el control integral del SM.

Con base en lo anterior surge la siguiente pregunta de investigación, ¿Las subfracciones de *Cucumis sativus* contrarrestan la disfunción en adipocitos?

4. Hipótesis

Las subfracciones de *Cucumis sativus* contrarrestan la resistencia a la insulina en adipocitos disfuncionales

5. Objetivo general

Evaluar el efecto de las subfracciones de *Cucumis sativus* sobre la resistencia a la insulina en adipocitos disfuncionales *in vitro* e *in vivo*

6. Objetivos particulares

- Identificar las subfracciones efectivas de Aq-Cs y su concentración para el control de la resistencia a la insulina de adipocitos 3T3-L1 disfuncionales.
- Evaluar las mezclas de las subfracciones efectivas de Aq-Cs para controlar la expresión de IL-6 y de O_2^- por adipocitos 3T3-L1 disfuncionales.
- Identificar los compuestos mayoritarios presentes en las subfracciones efectivas del Aq-Cs
- Evaluar *in vivo* la combinación efectiva de las subfracciones del Aq-Cs para el control de la resistencia a la insulina y expresión de interleucinas pro-inflamatorias en adipocitos disfuncionales en condición de obesidad

7. Metodología

7.1 Fraccionamiento de la fracción acuosa de *Cucumis sativus*

Las partes aéreas de Cs, incluido el fruto, se recolectaron de un cultivo comestible libre de pesticidas y fertilizantes en Xochitepec, Morelos, México, en el periodo de julio a agosto. El material vegetal se mantuvo alejado de la luz a temperatura ambiente y se secó en un horno a 50 °C durante 36 h. El material de la planta seca se trituró en un molino eléctrico hasta que se obtuvieron partículas de menos de 4 mm de diámetro. Este polvo se maceró con etanol/agua (60:40) a temperatura ambiente. El HA-CS se concentró a presión reducida y luego se liofilizó. El extracto (50 g) se fraccionó por cromatografía líquido-líquido usando una mezcla 1:1 de acetato de etilo/agua. La fase orgánica se sometió a un proceso de destilación a baja presión para eliminar el acetato de etilo y las partículas remanentes de este disolvente se eliminaron mediante extracción a alto vacío. El volumen de la fracción acuosa (Aq) se redujo por destilación a baja presión obteniendo una muestra semisólida que finalmente se secó mediante un proceso

de liofilización (Figura 4A). Esta última mezcla (Aq-Cs, 35 g) se suspendió en metanol (700 ml) durante 24 h, después de la filtración, la fase soluble se concentró en un evaporador rotatorio (Laborota 4000, Heidolph, Schwabach, Alemania), para obtener la subfracción SF1 (13,3 g). El precipitado resultante se resuspendió en acetona (700 ml) obteniéndose una solución verde que se concentró por destilación a baja presión obteniendo la subfracción SF2 (4,7 g) y un precipitado amarillo el cual se le denominó subfracción SF3 (3,2 g) (Figura 4B).

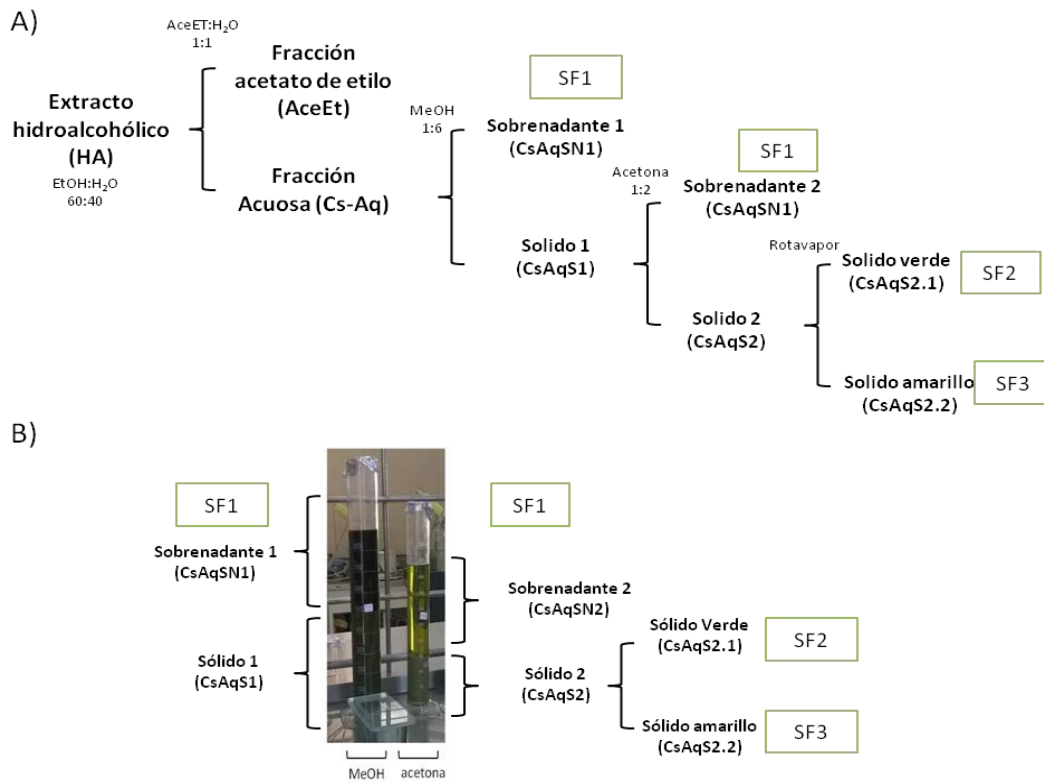


Figura 4. Fraccionamiento del Aq-Cs. El extracto HA-Cs fue diluido con Agua:acetato de etilo (1:1), se obtuvo las fracciones acuosa (Aq-Cs) y acetoetilico (AE-Cs). El Aq-Cs se diluyó con 1:6 metanol (MeOH), el sólido precipitado se diluyó con acetona en una relación 1:2 (v/v). El sobrenadante fue definido como SF1 y el precipitado formado fue subfraccionado y se obtuvo las SF2 y SF3.

7.2 Cuantificación de LPS en las subfracciones obtenidas a partir del Aq-Cs

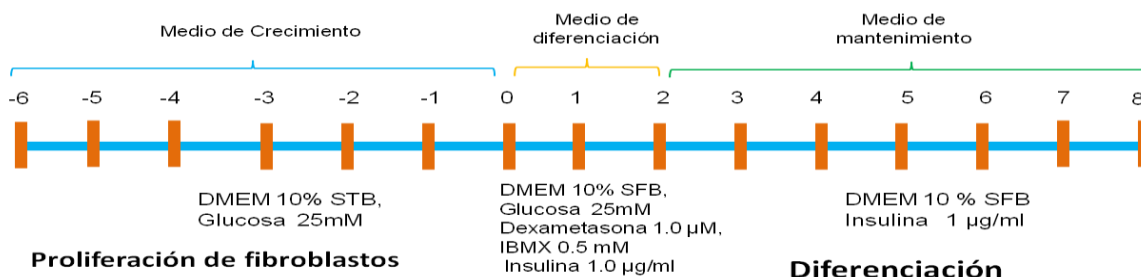
Con la intención de evaluar la presencia de LPS en las subfracciones obtenidas a partir del Aq-Cs, se utilizó la prueba cromogénica de lisados de amebocitos Limulus (LAL) CAMBREX pyrogen® plus (QLC-1000™). En el cual se usan lisados de amebocitos de

Limulus, que al interactuar con los LPS se produce la escisión de un sustrato sintético y liberan para-nitrolinina (pNA) la cual genera un color amarillento, siendo la concentración de pNA proporcional a la concentración de LPS. Para ello se incubó 50 μ l de las subfracciones de Aq-Cs con 50 μ l de una solución de LAL a 37°C durante 10 minutos. Pasado el tiempo de incubación, se agregaron 100 μ l de la solución de sustrato y se incubó durante 6 minutos a 37°C. Al término de la incubación, la reacción fue detenida con 100 μ l de la solución de paro (ácido acético, 25% v/v en agua), y la absorbancia fue determinada a una λ 405-410 nm en un lector de microplacas VERSAmax (Molecular Devices, Sunnyvale, CA, EE.UU.). La concentración de endotoxina fue calculada a partir de una curva estándar.

7.3 Cultivo celular

Los fibroblastos murinos 3T3-L1 se adquirieron de la American Type Culture Collection (ATCC CL-173; Rockville, MD) y se cultivaron en medio de Eagle modificado con Dulbecco de alta glucosa (DMEM) (ATCC 30-2002) suplementado con 10% suero bovino de ternera (Corning 35-022-CV, NY), gentamicina 20 μ g/ml (Gibco 15710-64, Grand Island, NY) a 37 °C en atmósfera de CO₂ al 5%, y se incubó durante 48 h. En el segundo día después de la confluencia, los fibroblastos se diferenciaron en adipocitos con dexametasona 1,0 μ M (Sigma D1881, St. Louis, MO), metilisobutilxantina 0,5 mM (MIBX, Sigma 15879) y 1,0 μ g/ml de insulina (Sigma 1882) en DMEM más 10% de suero bovino fetal (FBS, Gibco 16000-069). Dos días más tarde, el medio se reemplazó por DMEM fresco más 10% de SFB y 1,0 μ g/ml de insulina, y las células se dejaron madurar durante 6 días. Las células se procesaron cuando el 95% de ellas mostraron rasgos típicos de adipocitos (día 8) (Fortis 2012).

Línea de tiempo de diferenciación a adipocitos:



Una vez establecida la disfunción en los adipocitos como se describe a continuación, se agregaron cinco concentraciones (5, 10, 20, 40 u 80 µg / ml) de las tres subfracciones de la Aq-Cs (SF1, SF2 o SF3) al cultivo y se dejaron reposar por 24 h. Se incluyeron silimarina 50 µg/ml o metformina 1 mM. La silimarina se usó como control antioxidante y antiinflamatorio, mientras que la metformina se usó como control antihiper glucémico.

Posteriormente se realizó la evaluación antiinflamatoria y antioxidante de las combinaciones de las fracciones efectivas, las cuales se muestran en la siguiente tabla:

Tabla 3: Combinaciones obtenidas del modelo de superficie

	SF1	SF2	SF3
M1	2.5 µg/ml	20 µg/ml	7.5 µg/ml
M2	2.5 µg/ml	20 µg/ml	2.5 µg/ml
M3	2.5 µg/ml	20 µg/ml	5 µg/ml
M4	40 µg/ml	60 µg/ml	30 µg/ml
M5	40 µg/ml	60 µg/ml	10 µg/ml

Para evaluar la viabilidad celular a través del ensayo MTT y realizar los ensayos de consumo de 2-desoxiglucosa (2-DG), se sembraron adipocitos a una concentración de $1,5 \times 10^3$ células/pozo en placas de 96 pozos. Para evaluar la liberación de glicerol, la concentración de IL-6 y la presencia de O_2^- , las células se sembraron en una concentración de $4,5 \times 10^4$ células/pozos en placas de 12 pozos y se procesaron posteriormente.

7.4 Evaluación de viabilidad celular por MTT

Con la intención de evaluar el efecto de las subfracciones sobre la viabilidad celular, los adipocitos maduros fueron incubados durante 24 horas con diferentes concentraciones de las tres subfracciones del Aq-Cs (5, 10, 20, 40 o 80 µg/ml), silimarina (50 µg/ml), Metformina (1 nM) o DMSO 60%. Después del tiempo de incubación, se agregó 5 mg/ml de una solución de Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazolio (MTT, sigma, M 2128) y se incubó en oscuridad a 37°C, pasado el tiempo de incubación se agregaron 100 µl de una solución de SDS 10% con HCl 0.01 N (1:1) a cada pozo y se incubaron 2 horas más a 37°C. Al término de la incubación se cuantificó la absorbancia a una λ de 570 nm en un lector de microplacas VERSAmax (Molecular Devices, Sunnyvale, CA, EE.UU.). El porcentaje de las células viables fue calculado utilizando la siguiente fórmula:

$$\% \text{ Viabilidad} = \frac{\text{DO células tratadas} * 100}{\text{DO células control}}$$

7.5 Inducción de adipocitos 3T3-L1 disfuncionales

Con la intención de evaluar la capacidad de las subfracciones para controlar la disfunción en adipocitos. Los adipocitos maduros se incubaron durante 24, 48, 60 o 72 h con 0,1 µM o 1 µM de dexametasona, con o sin insulina 1 µM. En otro experimento, para inducir adipocitos disfuncionales, las células se cultivaron en presencia de 1 ng/ml de IL-1β (DB Biosciences 51-26666E, San Diego, CA) durante 24, 48, 60 o 72 h, con o sin Insulina 1 µM. Como se mencionó anteriormente, la densidad celular final utilizada dependía del ensayo que se realizaría.

7.6 Consumo de 2-Deoxiglucosa

El consumo de 2-DG se determinó para seleccionar las condiciones más eficientes para inducir adipocitos disfuncionales, y para evaluar la efectividad de las

concentraciones de las subfracciones de la Aq-Cs evaluadas para controlar la disfunción metabólica. El consumo de 2-DG se determinó mediante un ensayo colorimétrico con el kit Uptake 2-DG (Sigma MAK083), siguiendo las instrucciones del fabricante. Brevemente, las células se lavaron dos veces con Buffer de fosfatos salino (PBS) y se incubaron en DMEM sin FBS durante 4 h. Las células se lavaron tres veces y se incubaron durante 40 min en buffer KRPH (HEPES 20 mM, KH₂PO₄ 5 mM, MgSO₄ 1 mM, CaCl₂ 1 mM, NaCl 136 mM y KCl 4,7 mM) más albúmina bovina al 2%. Posteriormente, las células se estimularon con insulina 1 μM durante 20 minutos; se añadió 10 mM 2-DG, se mezcló y se incubó durante 20 minutos. Las células fueron lisadas en ciclos de calentamiento-congelación, y se añadió la solución de reacción A (buffer de ensayo más mezcla de enzimas en una proporción de (8: 2)); las placas se incubaron a 37 ° C durante 60 minutos en la oscuridad; luego, se añadió buffer de extracción y se dejó reposar durante 5 minutos, y la reacción se detuvo añadiendo la solución de reacción B (53% de glutatión reductasa, 42% de sustrato DTNB y 5% de mezcla de reciclado). Las placas se mezclaron completamente y la absorbancia se midió a 412 nm. La concentración de 2-DG fue calculado a partir de una curva estándar.

7.7 Cuantificación de glicerol en el medio de cultivo

La liberación de glicerol fue determinada para seleccionar las condiciones óptimas para inducir la disfunción de los adipocitos y para evaluar la eficacia de las concentraciones de las subfracciones de la Aq-Cs evaluadas para revertir la disfunción. La concentración de glicerol se cuantificó en el medio usando el kit Colorimetric Assay Glycerol (Sigma MAK117). Brevemente, se transfirieron 10 μl de medio de cultivo de cada condición a placas de 96 pozos y se añadieron 100 μl/pozo de Master Reaction Mix; después, las placas se incubaron durante 20 minutos a temperatura ambiente, en oscuridad. Luego, se midió la absorbancia a 570 nm. La concentración de glicerol fue calculado a partir de una curva estándar usando la siguiente ecuación:

$$C=(A_{570})\text{muestra}/\text{pendiente}$$

C: Concentración de glicerol en mM

Pendiente: Pendiente determinada por la curva estándar

7.8 Modelo de superficie de respuesta

Se construyó un modelo de superficie de respuesta para determinar la combinación de las concentraciones efectivas de las subfracciones que puedan controlar a los adipocitos disfuncionales. El diseño experimental consistió en evaluar el efecto de las variables X_1 , X_2 y X_3 , que corresponden a concentraciones efectivas de las subfracciones SF1, SF2 y SF3 en dos variables de respuesta Y_1 e Y_2 (consumo de 2-DG y liberación de glicerol). Esto, debido a que las concentraciones de las subfracciones de la Aq-Cs que aumentaron el consumo de 2-DG fueron de 40 $\mu\text{g/ml}$ (SF1 y SF2) y 20 $\mu\text{g/ml}$, y las concentraciones que disminuyeron la liberación de glicerol fueron de 5 $\mu\text{g/ml}$ (SF1 y SF3) y 40 $\mu\text{g/ml}$ (SF2). El diseño factorial se ajustó a 2^{3-1} modelos polinómicos cuadráticos, con cinco combinaciones en total (M1-M5). Las variables fueron codificadas acorde a la siguiente ecuación:

$$x_i = (X_i - X_0)/\Delta X_i$$

Donde x_i es el valor codificado para la variable independiente; X_i es el valor real de la variable independiente; X_0 es el valor de la variable independiente en el punto central, y Δx_i es el valor de incremento de la variable independiente.

El modelo predictivo para el punto óptimo fue expresado acorde a la siguiente ecuación:

$$Y_n = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j=1}^3 b_{ij} X_i X_j$$

Donde Y_n es la respuesta de las variables, b_0 es el coeficiente de regresión y X_i es el nivel codificado de cada variable independiente. Los datos fueron analizados aplicando el procedimiento de regresión para la superficie de respuesta, usando el software Minitab® v18.1. Tres niveles fueron codificados para las variables independientes, máximo, intermedio y el mínimo, la amplitud de cada nivel fue del 50%.

Tabla 2. Variables independientes experimentales y niveles usados

		Niveles			
	Variable	Codificado	Codificado		
			-1	0	1
Liberación de glicerol	SF1	X ₁	2.5 µg/mL	5 µg/mL	7.5 µg/mL
	SF2	X ₂	20 µg/mL	40 µg/mL	60 µg/mL
	SF3	X ₃	2.5 µg/mL	5 µg/mL	7.5 µg/mL
Consumo de 2-DG	SF1	X ₁	20 µg/mL	40 µg/mL	60 µg/mL
	SF2	X ₂	20 µg/mL	40 µg/mL	60 µg/mL
	SF3	X ₃	10 µg/mL	20 µg/mL	30 µg/mL

7.9 Cuantificación de citocinas mediante la técnica de ELISA en sándwich

Los adipocitos se trataron durante 24,48, 60 o 72 horas con dexametasona 1 µM o con IL-1β 1 ng/ml. Después de la incubación, el medio de cultivo fue recogido y la concentración de IL-6 fue cuantificada por ELISA (OptEIA™ BD, 555240, San Diego) siguiendo las instrucciones descritas por los fabricantes. Previamente se sensibilizaron placas de 96 pozos con 100 µl por pozo de anticuerpo de captura a una concentración de 1:250 diluido en buffer de Carbonatos de sodio 0.1 M pH 9.5. Se incubó toda la noche a 4 °C. Al día siguiente se realizaron lavados con PBS-Tween 20 al 0.05%, los pozos fueron bloqueados con PBS-SFB 10% y se incubó durante una hora, al terminar el tiempo de incubación las placas se lavaron, se adiciono 100 µl de muestra y/o curva estándar a cada pozo y se incubó durante 2 horas, después la placa fue lavada y se agregó 100 µl por pozo de una concentración 1:500 del anticuerpo de detección en PBS-SFB 10% acoplado a la enzima HRP (1:250) y se incubó durante 1 hora, pasado el tiempo de incubación se realizaron lavados. La lectura de densidad óptica se realizó después de unos 10 a 30 minutos de incubación con el substrato TMB (Life technologies, 002023) y su posterior paro de reacción con H₂SO₄ 2 N en un lector de ELISA a 450 nm. La concentración de IL-6 fue calculada como pg/mg de proteína en una curva estándar. La cuantificación de proteínas se realizó mediante el método de Bradford (Sigma B6916-500ML).

7.10 Cuantificación de O_2^- a través de Dihidroetidio

El O_2^- se detectó mediante el uso de dihidroetidio (DHE) el cual se oxida a etidio por este ROS (Pedraza *et al.*, 2009). Brevemente, los adipocitos se cultivaron en placas de 12 pozos y se incubaron con o sin dexametasona $1 \mu\text{M}$ y/o insulina $1 \mu\text{M}$; o 1 ng/ml de IL- 1β y/o insulina $1 \mu\text{M}$ durante 24, 48, 60 o 72 h. Cinco minutos antes de finalizar el tiempo de incubación, se añadió DHE $20 \mu\text{M}$ (Sigma D7008). Luego, las placas se lavaron tres veces con PBS. Se añadió DMEM más 10% de FBS, y las células se analizaron y fotografiaron usando un lector de placas multimodal de imagen celular Cytation 5 (Biotek Instruments, Winooski, VT), usando el software Gen 5 (instrumentos Biotek) bajo un objetivo 20X. La intensidad de fluorescencia se cuantificó con el software MetaMorph Image Analysis v.6.1. (Molecular Devices, Sunnyvale, CA).

7.11 Determinación de compuestos presentes en las subfracciones por cromatografía en capa fina

Con la intención de conocer el perfil químico de las subfracciones obtenidas de la Aq-Cs, se realizó Cromatografía en Capa Fina (CCF) tanto de fase normal como de fase reversa. Para ambas, se utilizó una fase estacionaria recubierta por sílica gel (Cromatofolio de sílica gel 60 F₂₄₅, Merck), a la que se le marcaron los puntos de aplicación de la muestra con un espacio de 0.5 cm en el borde inferior. Posteriormente se colocó la muestra y los estándares comerciales para flavonoides (Rutina), aminoácidos (glicina, alanina, serina, valina, leucina, asparagina, ácido aspártico, lisina, ácido glutámico y arginina) y polisacáridos (glucosa). Luego la placa con las muestras se colocó en una parrilla con la intención de eliminar el disolvente y se colocó en una cámara cromatográfica la cual ya contenía la fase móvil. Para la fase normal se utilizaron diversos sistemas de diferente polaridad, entre ellos Diclorometano:metanol (7:3 v/v), acetato de etilo:metanol:agua:ácido acético glacial (7:2:2:0.5 v/v) y n-butanol:acetona:ácido acético glacial:agua (35:35:10:20 y 70:70:20:10 v/v), mientras que para la fase reversa se utilizó un sistema de agua:acetonitrilo (7:3 v/v). Las placas se retiraron de cada sistema cuando alcanzaron el 90% de corrimiento de manera que quedara un espacio de 0.5 cm entre el

límite de la corrida y el final superior. Las placas se acomodaron en la parrilla para eliminar el solvente y se observaron bajo luz UV. Posteriormente a las placas se les aplicaron los reveladores Naftol (Sigma) para la determinación de polisacáridos, Ninhidrina (Merck) para determinar aminoácidos, 4-hidroxibenzaldehído (Merck) o revelador para flavonoides (difenilborato de aminoetanol) (Sigma) siguiendo las instrucciones del proveedor. Una vez reveladas las placas fueron secadas en la parrilla, se observaron los patrones de manchas bajo luz UV y luz visible, y se compararon entre fracciones.

7.12 Análisis ULPC-MS

Para identificar los compuestos bioactivos, las subfracciones SF1, SF2 y SF3 se sometieron a un análisis cromatográfico utilizando un Acquity UPLC (Waters, Milford MA, EE. UU.), el cual cuenta con una bomba cuaternaria y una columna con inyector automático. La cromatografía de líquidos se realizó a 30° C, utilizando una columna Acquity UPLC BEH 1.7 C18 (2.1 x 10 mm, 1.7 mm). Dicha columna se eluyó con un sistema de gradiente que consistía en ácido fórmico al 0,1% en agua (A) y ácido fórmico al 0,1% en acetonitrilo (B) con un caudal de 0,3 µl/min. La columna se mantuvo al 100% de A durante 2 minutos y posteriormente se elevó al 100% de B (curva 6) durante 14 minutos, seguido de un período de 1 minuto al 100% de B, antes de un rápido retorno al 100% de A, y un período de equilibrio de 2 min. El tiempo de corrida fue de 20 minutos. El volumen de inyección de las muestras fue de 5 µL. El análisis de espectrometría de masas se realizó y analizó en un espectrómetro de masas TQD de triple cuadrupolo (Waters, Milford, MA) a través de una fuente de iones de electropulverización Z-spray, en un modo ESI positivo. Las temperaturas de fuente y desolvatación fueron de 150 y 450° C respectivamente. Se utilizó una combinación de voltaje de cono de 20V y voltaje capilar de 2.5 kV. Se empleó nitrógeno tanto para el gas de desolvatación como para el gas de cono. Las condiciones óptimas de detección se determinaron mediante la infusión constante de soluciones estándar (50 µM) en disolvente. La exploración de MS se realizó utilizando argón como gas de colisión. Las condiciones de detección se determinaron mediante infusión constante de soluciones estándar (50 µg/ml) en agua acidulada (ácido

trifluoracético al 0.05%). Para identificar los principales aminoácidos contenidos en cada fracción se utilizaron como estándares cromatográficos los estándares comerciales de glicina (6-7126), arginina (A-4881), asparagina (A-8131), lisina (L-6001), leucina (L-7750), isoleucina (I-2627), aspartato (A-8881) y glutamato (6-1001) (todo de sigma).

7.13 Inducción de obesidad murina

Se utilizaron ratones machos de la cepa C57BL/6J de 18 a 20 gramos que se alimentaron con una dieta hipercalórica (DHC) (Harlan TD.06414 Adjusted Calories Diet (60/Fat)) de manera crónica. Al inicio del experimento, los ratones fueron evaluados de manera individual en cuanto a peso, índice de Lee (proporcional al índice de masa corporal en humanos); así como glicemia, colesterol y triglicéridos en ayunas, curvas de tolerancia a la glucosa e insulina, lo que se repitió a las 15 semanas de ingesta de la dieta, momento en que los ratones presentaban obesidad. Posteriormente, la administración de la combinación M5 se llevó a cabo durante 10 semanas más, en donde al final se evaluó el efecto de esta combinación sobre los parámetros metabólicos y físicos. El manejo de los animales de laboratorio se realizó con estricto apego a la NOM vigente (Norma Oficial Mexicana NOM-062-ZOO-1999, especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio).

7.14 Grupos experimentales

Los ratones fueron agrupados de acuerdo al tratamiento que recibieron. Los ratones del grupo control ingirieron la dieta isocalórica (DIC) durante 25 semanas. Dos grupos más fueron alimentados con una dieta hipercalórica (DHC) también por 25 semanas, para el desarrollo de obesidad. A las 15 semanas de alimentación con la DHC a un grupo de ratones se le administró vía oral de la combinación M5 (SF1= 40 µg/ml; SF2= 60 µg/ml; SF3= 10 µg/ml).

7.15 Medición del peso

Los ratones fueron pesados al inicio de la ingesta de la DHC (semana 0), y al inicio de la administración de la M5 (semana 15) y al final de la administración de la M5 (semana 25).

7.16 Determinación del índice de Lee

Una vez pesados los ratones, se determinaron las longitudes naso-anales para calcular el índice de Lee de obesidad que es proporcional al índice de masa corporal en humanos, usando la fórmula:

$$\text{Índice de obesidad de Lee} = \text{peso (g)} \times 0.33 / (\text{longitud naso-anal (cm)})^2$$

El índice de Lee, se calculó en las mismas semanas en que fueron pesados los ratones, semana 0, 15 y 25.

7.17 Determinación sérica de glucosa en ayunas y curva de tolerancia a la glucosa

Para la determinación sérica de glucosa al inicio del experimento (semana 0), al intermedio (15 semanas) y al final del experimento (25 semanas), se tomó una muestra de sangre periférica (de la vena caudal) después de un ayuno de 6 horas. Para la curva de tolerancia a la glucosa, se tomó una muestra inicial como se explicó anteriormente, que será el basal, y posteriormente se administró oralmente una solución de glucosa con una concentración de 2 g/Kg de peso del ratón. Se tomó muestras de sangre cada 30 min hasta los 120 min. Las determinaciones se realizaron con un glucómetro de la marca Accu-chek Performance de Roche. Con los datos obtenidos se construyó la curva de respuesta y se calculó el área bajo la curva mediante el método del Trapezoide, a través de la siguiente fórmula.

$$\sum_{x_0} x_n(x) \delta X = 1/2 h [(y_0 + y_n) + 2(y_1 + y_2 + y_3 + y_{n-1})]$$

7.18 Determinación indirecta de la resistencia a la insulina

Para la determinación de manera indirecta de la resistencia a la insulina, se realizó una curva de tolerancia a la insulina. En la cual los ratones se sometieron a 4 horas de ayuno y se obtuvo una muestra de sangre periférica (vena caudal) de los animales, se cuantificó la concentración de glucosa (mg/dl) utilizando un glucómetro (tiempo 0) y posteriormente se administró insulina humana de acción rápida (Humulin-R Lilly France, S.A., Francia) vía intraperitoneal a una concentración de 0.25 UI/Kg y se cuantificó nuevamente la glucemia a los 15, 30, 60, 90 y 120 minutos posteriores a su aplicación.

7.19 Determinación sérica de triglicéridos y colesterol en ayunas

Con la intención de evaluar el efecto tanto de la ingesta de la DHC (semana 15) así como de la administración de la M5 (semana 25) sobre la concentración sérica de triglicéridos y colesterol. Se obtuvo una muestra de sangre de la vena maxilar, la cual fue centrifugada a 1500 rpm durante 8 minutos y se obtuvo el suero. Para cuantificar estos parámetros se utilizaron los kits comerciales de Química clínica aplicada S.A.: Colesterol-LQ (990159) y triglicéridos-LQ (41031), siguiendo las instrucciones del fabricante. Para ello, se agregaron 250 µl/pozo del reactivo de color correspondiente a cada parámetro a evaluar (colesterol o triglicéridos, según el kit), y se adicionaron 2.5 µl de la muestra a evaluar. Se dejó incubar durante 10 minutos a temperatura ambiente en oscuridad y posteriormente se realizó la lectura de la absorbancia a 505 nm en un lector de microplacas VERSAmax (Molecular Devices, Sunnyvale, CA, EE.UU.).

Los cálculos se realizaron mediante la siguiente fórmula:

A: absorbancia

$$\frac{A \text{ muestra}}{A \text{ estándar}} = \text{x concentración del estándar} = \text{mg/dl colesterol o triglicéridos}$$

7.20 Determinación de citocinas en extractos acuosos tisulares

Con la intención de evaluar el efecto antiinflamatorio de la M5, después de las 10 semanas de la administración de la M5 se obtuvo hígado y tejido adiposo subcutáneo (TAS). Los cuales fueron procesados para obtener extractos acuosos tisulares en presencia de un inhibidor de proteasas de serina el PMSF al 0.1% en una dilución 1:10 con respecto al peso del órgano o tejido. Posteriormente se cuantificó la concentración de citocinas proinflamatorias y antiinflamatorias en dichos extractos acuosos tisulares, mediante la técnica de ELISA, siguiendo el protocolo descrito anteriormente y usando los siguientes kits: IL-10, IL-6, TGF- β e IL-1 β , todos de BD OptEIA.

7.21 Análisis estadístico

Todos los parámetros se recopilaron en una base de datos utilizando el programa Excel. Las diferencias entre grupos se realizaron con una prueba de ANOVA y una prueba a posteriori de Tukey-Kramer. Todos los datos se analizaron con el programa INSTAT para realizar los análisis uni y multivariados. Con un valor de $P \leq 0.05$.

8. Resultados

8.1 La concentración de LPS presente en las subfracciones de la Aq-Cs son menores a 4 UE/ml.

El LPS es el mayor componente glucolípido de la membrana exterior de las bacterias gram negativas, está conformado por cadenas de oligosacáridos y polisacáridos, así como una parte lipídica (lípidos A), este último al unirse al TLR4 inicia una señalización en cascada que resulta en la activación de una respuesta pro-inflamatoria y un aumento de estrés oxidante (Nabil *et al.*, 2015). Saber su concentración en las subfracciones, era importante debido a que se ha observado una relación entre el desarrollo de la metainflamación en tejido adiposo durante la obesidad con la concentración de LPS (Hersoug *et al.*, 2018).

En la figura 5, se muestran las concentraciones de LPS representadas por unidades de endotoxinas por ml (EU/ml), presentes en los tratamientos, en donde se puede observar que el agua inyectable (blanco) (0.1 EU/ml), el agua destilada utilizada para la preparación de las subfracciones (0.3 EU/ml) y la SF3 (0.2 EU/ml), no presentan diferencias significativas ($p > 0.05$) indicando la ausencia de LPS. Por otro lado, las subfracciones SF1 y SF2 tienen un 2.8 y 3.2 EU/ml, respectivamente, que, aunque son significativamente más altos que el control negativo ($p \leq 0.05$) se encuentran dentro de los valores que no inducen una respuesta pro-inflamatoria (Ying *et al.*, 2000).

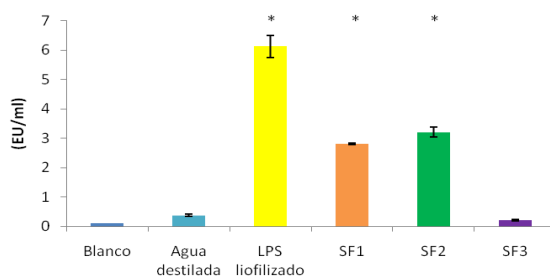


Figura 5. Cuantificación de LPS. Se realizó el ensayo LAL, el cual cuantifica la concentración de unidades de endotoxinas por ml (EU/ml), en el agua destilada, LPS liofilizado, SF1, SF2 y SF3. Los valores son representados como media \pm DE. Los valores con significancia estadística son considerados con un valor de $P \leq 0.05$. Los asteriscos indican diferencias estadísticas significativas.

8.2 Las subfracciones obtenidas a partir de la Aq-Cs no afectan la viabilidad de los adipocitos evaluada por MTT

La determinación del efecto de las subfracciones sobre la viabilidad en el adipocito es un requisito crítico para analizar su capacidad para controlar la resistencia a la insulina y/o las condiciones pro-inflamatorias y pro-oxidantes inducidas por dexametasona o IL-1 β . Como se muestra en la Fig. 6, todas las concentraciones de las subfracciones evaluadas (5, 10, 20, 40 u 80 μ g/ml) arrojaron valores de viabilidad celular que oscilan entre 74,4% y 100% con respecto a adipocitos metabólicamente sanos (Adipocito con 1 μ M Insulina); en contraste, el tratamiento con 60% de DMSO causó una disminución significativa del 89.5% en la viabilidad de los adipocitos con respecto a las células sanas ($P \leq 0.05$).

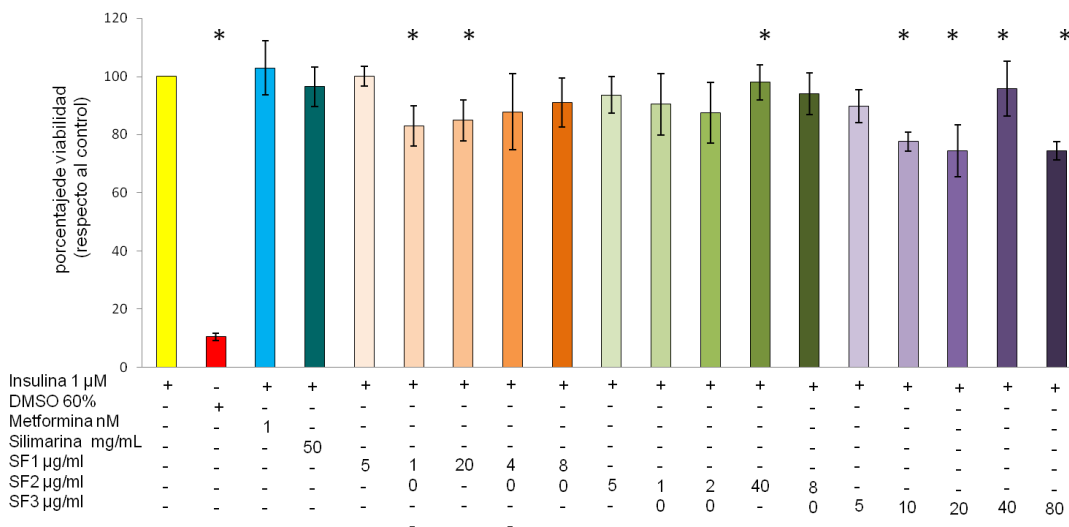


Figura 6. Efecto de las subfracciones de la CsAq sobre la viabilidad de las células 3T3-L1. Las células 3T3-L1 fueron tratadas con 0.016, 0.08, 0.4, 2 o 10 μ g/ml de las SF1, SF2, SF3 y con DMSO 60% durante 24 horas. Otras células no recibieron tratamiento. La viabilidad celular se evaluó por el ensayo de MTT. Los valores son representados como promedio \pm DE. Los asteriscos indican diferencias estadísticas significativas ($p \leq 0.05$).

8.3 En adipocitos maduros se induce disfunción con 1 μ M de insulina y 1 μ M de dexametasona

Se ha demostrado que una administración constante de glucocorticoides como la dexametasona ha demostrado que induce resistencia a la insulina, la cual es una característica clave de la disfunción. En adipocitos en cultivo, la resistencia a la insulina se observa como una disminución en el consumo de glucosa y/o la incapacidad para almacenar lípidos. Como se muestra en la Fig. 7A, la dexametasona 0.1 μ M no alteró el consumo de 2-DG (un análogo de glucosa) a las 24 horas de incubación, mientras que la dexametasona 1 μ M disminuyó significativamente (14.3%, $P \leq 0.05$) el consumo de 2-DG con respecto a los adipocitos sin insulina (Fig. 7A). Después de 48 h, se observó una disminución significativa ($P \leq 0.05$) de 45.8% (0.1 μ M) y 43.3% (1 μ M) con respecto al control. Finalmente, no se observaron diferencias significativas en el consumo de 2-DG con respecto al control solo a las 60 y 72 h de incubación.

La concentración de glicerol en el medio se cuantificó como otro marcador de la disfunción de los adipocitos. No se observaron diferencias significativas a las 24 h de incubación con 0.1 μ M o 1 μ M de dexametasona ($P > 0.05$) con respecto a los adipocitos

tratados sólo con insulina (Fig. 7B). Mientras tanto, se observó un aumento significativo ($P \leq 0.05$) de 54%, 40.1% y 25.2% en células tratadas con dexametasona 0.1 μM a 48, 60 y 72 h de cultivo, respectivamente. La liberación de glicerol aumentó un 67.8% y un 63.4% y disminuyó un 37.4% en los adipocitos tratados con dexametasona 1 μM en comparación con los controles a las 48, 60 y 72 h, respectivamente. Estos resultados indican que las células tratadas con 1 μM de dexametasona más 1 μM de insulina y 0.1 μM de dexametasona más 1 μM de insulina durante 48 h indujeron insensibilidad a la glucosa y aumentan la lipólisis como manifestación de la disfunción de los adipocitos.

Por otro lado, se evaluó la capacidad de la dexametasona para inducir un estado pro-inflamatorio y un estado pro-oxidante; sin embargo, la dexametasona no indujo un aumento significativo en la secreción de IL-6 con respecto a los adipocitos con insulina en los tiempos evaluados (Fig. 7C). Y se obtuvo un resultado similar en la cuantificación de O2- (Figura 8).

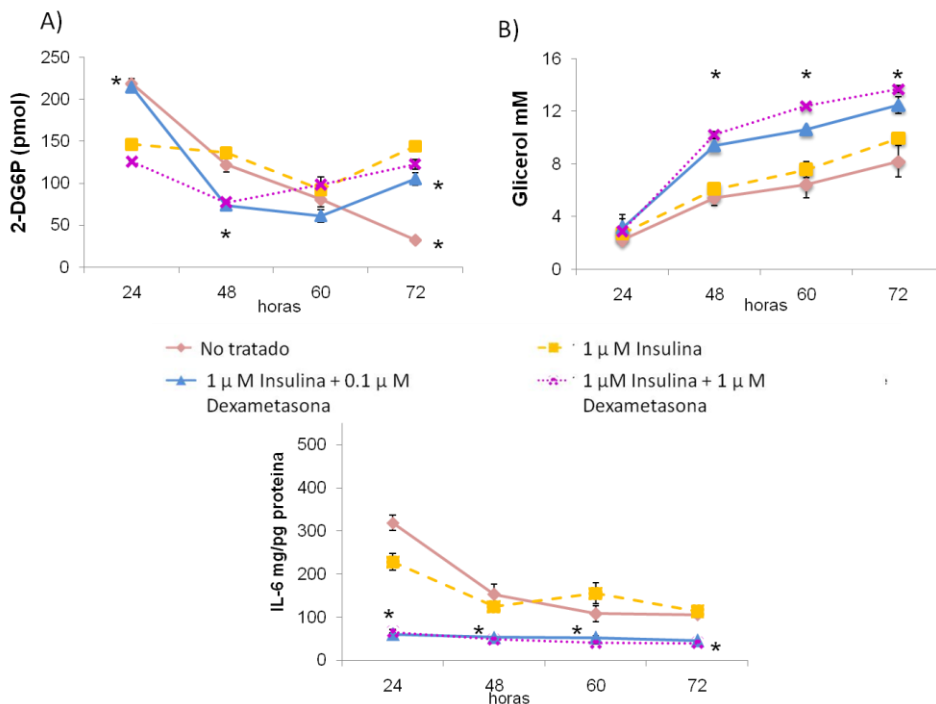


Figura 7. Cinética de inducción de disfunción por Dexametasona. Se incubaron adipocitos con 0.1 μM y 1 μM durante 24, 48, 60 o 72 horas y se cuantificó: A) consumo de 2-DG; B) Concentración de glicerol; y C) Concentración de IL-6 en el medio de cultivo. Los valores son representados como media \pm DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$).

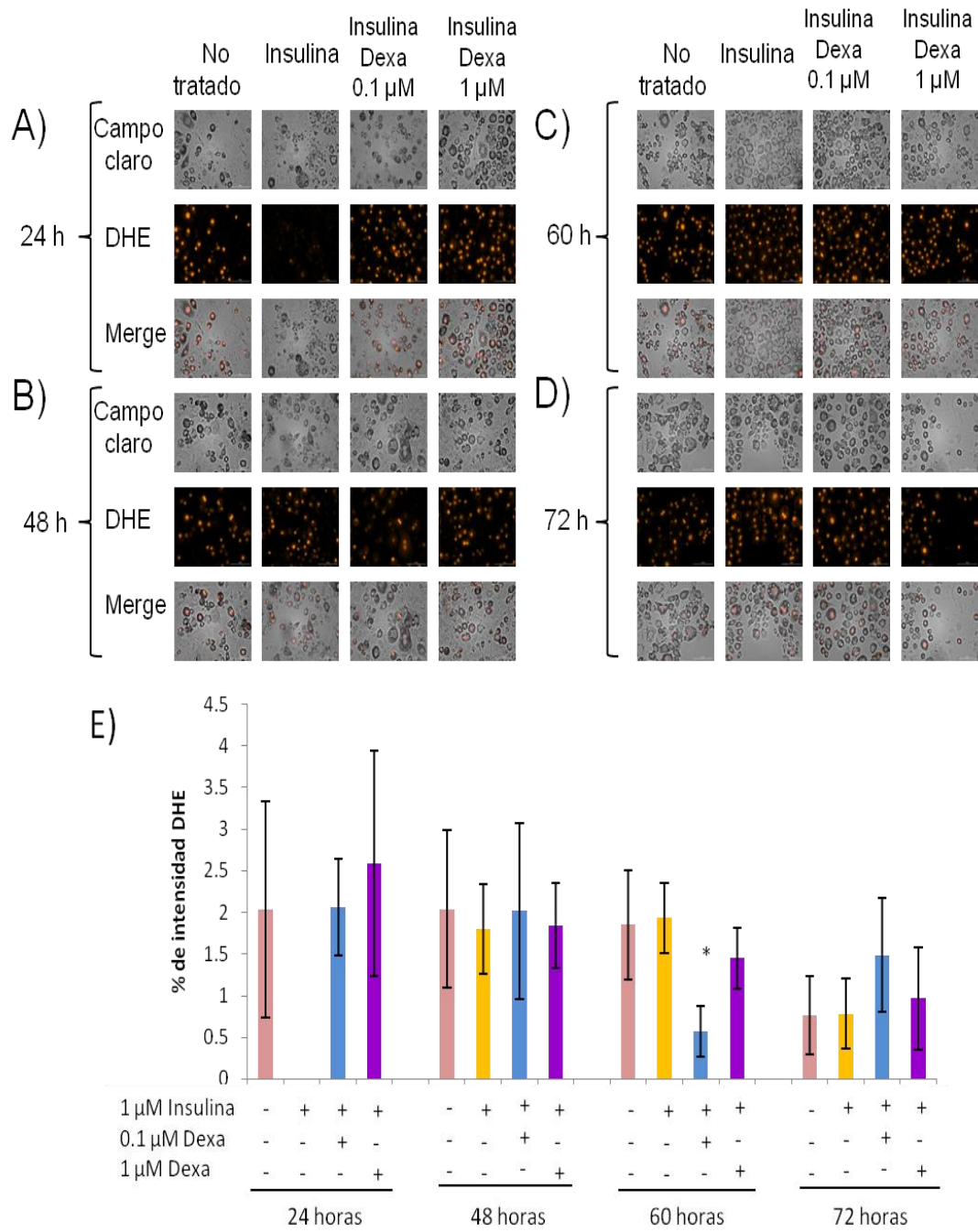


Figura 8. Expresión de DHE inducido por Dexametasona. Se incubaron adipocitos con 0.1 μ M y 1 μ M durante 24, 48, 60 o 72 horas y se cuantificó el % de intensidad de DHE. Los valores son representados como media \pm DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$).

8.4 En adipocitos maduros la IL-1 β (1ng/ml) aumenta la expresión de IL-6 y la producción de O $_2^-$

Los adipocitos disfuncionales se caracterizan no solo por una disminución en el consumo de glucosa y un almacenamiento inadecuado de lípidos, sino también por un estado pro-inflamatorio (aumento de las concentraciones de IL-6) y estrés oxidativo (aumento de los niveles de O $_2^-$ citosólico) (Fortis *et al.*, 2013; Rotter *et al.*, 2003). Y dado que la presencia de dexametasona no indujo esas condiciones, se evaluó la capacidad de la IL-1 β para inducir un aumento en la secreción de IL-6 y en la expresión de O $_2^-$.

En los resultados se observó un aumento significativo, casi del doble ($P \leq 0,05$) en la secreción de IL-6 cuando los adipocitos se cultivaron solos y con IL-1 β con o sin insulina durante 24 h (Fig. 9A). A las 48 horas se observó que la presencia de la IL-1 β ya sea en presencia o ausencia de la insulina hay un aumento significativo en la concentración de IL-6 en los tiempos evaluados a las 48 horas (aumento de 2,1 y 1,7 veces), 60 horas (aumento de 2 y 1,3 veces) y 72 horas (aumento de 2,8 y 1,4 veces).

Como se muestra en la Fig. 9B, se observó un aumento significativo ($P \leq 0.05$) en la intensidad de DHE (aproximadamente 50% en intensidad de DHE, Fig. 10) en adipocitos tratados con insulina o con IL-1 β durante 24 h, indicando niveles mayores de O $_2^-$. Por otro lado, el tratamiento con IL-1 β durante 48 h causó un aumento significativo de aproximadamente el 50% ($P \leq 0.05$) en la intensidad del DHE con respecto a las células con insulina (figs. 10B y 10E). A las 60 h de cultivo, se observó un aumento similar de aproximadamente 50% ($P \leq 0,05$) en la intensidad del DHE en células tratadas sólo con insulina, y también en aquellas tratadas con IL-1 β (Figuras 10C y 10E).

Estos resultados indican que la secreción de IL-6 y la producción de ROS aumentaron en los adipocitos tratados con IL-1 β 1 ng/ml durante 48 h. Por lo tanto, esta condición se seleccionó para evaluar la capacidad de las combinaciones de las subfracciones de la Aq-Cs para controlar estos parámetros.

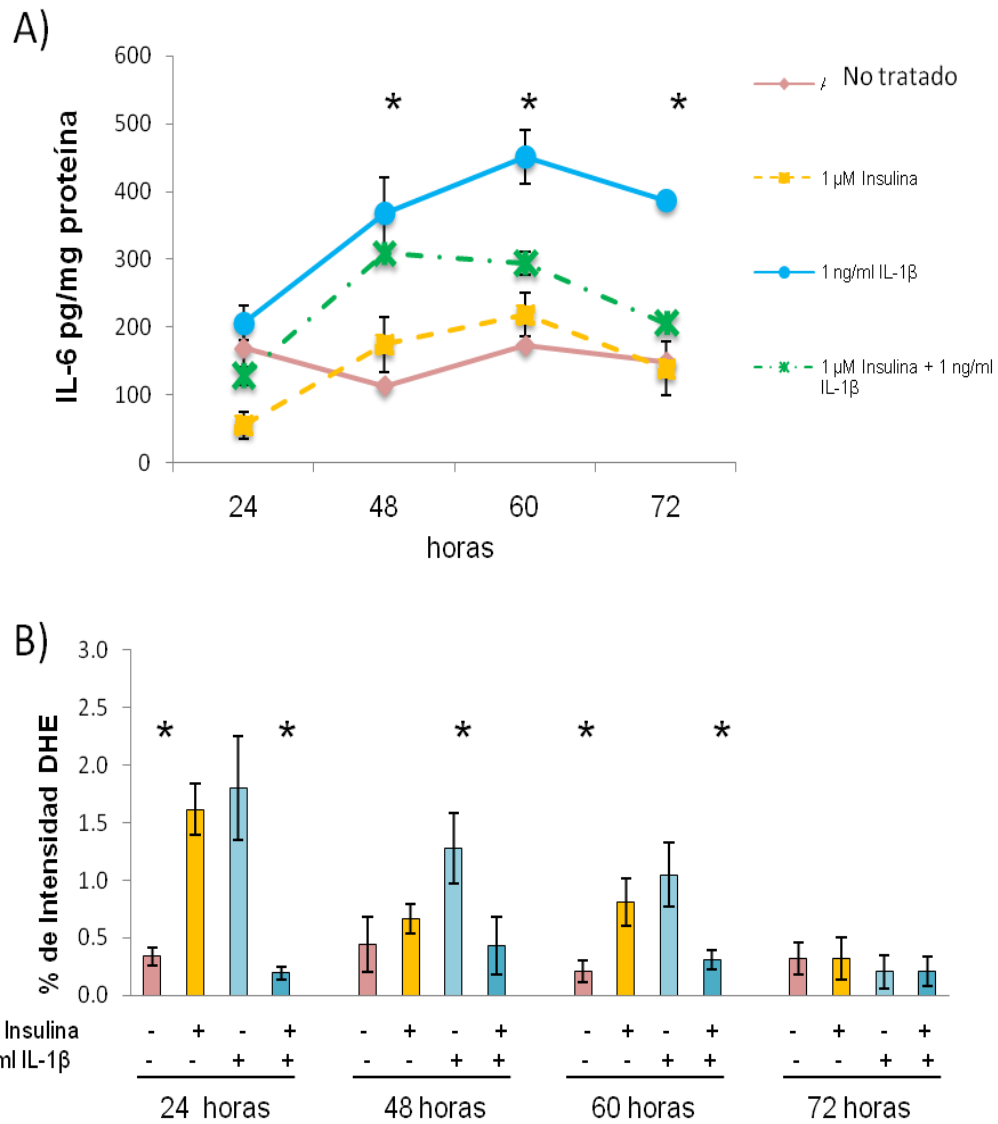


Figura 9. Cinética de inducción de disfunción por IL-1β. Se incubaron adipocitos con 1 ng/ml de IL-1β durante 24, 48, 60 o 72 horas, con o sin insulina y se cuantificó: A) La concentración de IL-6 en el medio de cultivo y B) el porcentaje de intensidad de DHE. Los valores son representados como media±DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$).

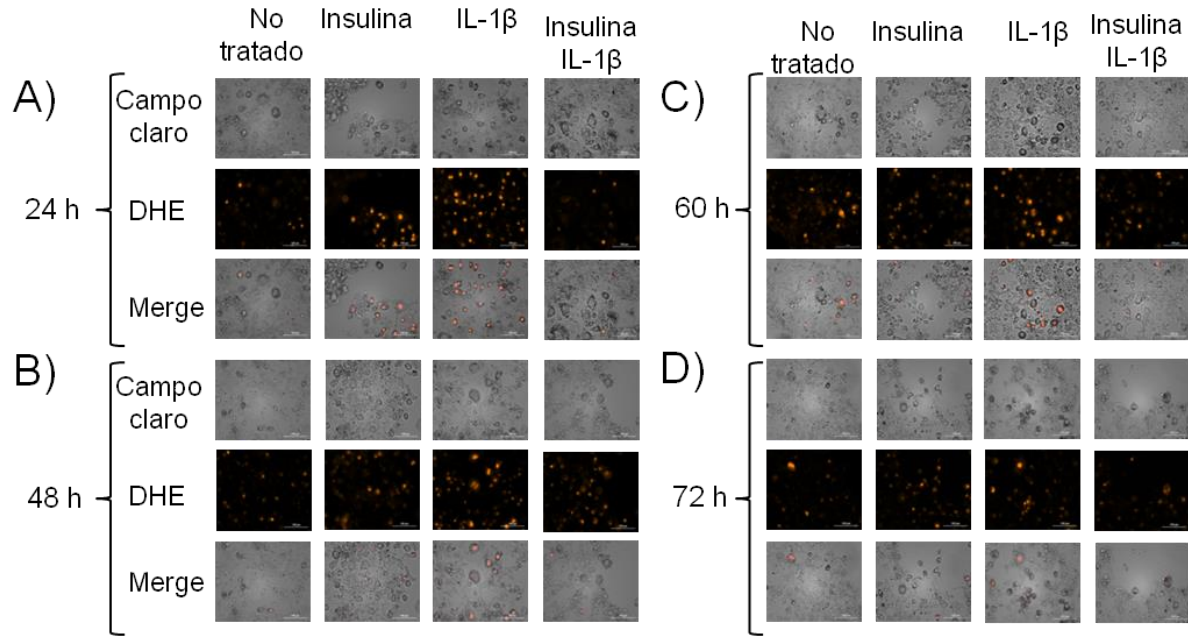


Figura 10. Expresión de DHE inducido por IL-1 β Se incubaron adipocitos con 1 ng/ml de IL-1 β con o sin insulina 1 μ M durante 24, 48, 60 o 72 horas y se cuantificó la expresión de DHE.

8.5 Las SF1 (5 μ g/ml), SF2 (40 μ g/ml) y SF3 (5 μ g/ml) disminuyen la concentración de glicerol en el medio de cultivo de adipocitos disfuncionales

Se indujo disfunción en los adipocitos mediante el cultivo de adipocitos maduros con dexametasona 1 μ M e insulina 1 μ M durante 48 horas. El efecto de las subfracciones de la Aq-Cs SF1, SF2 y SF3 se evaluó midiendo la liberación de glicerol, y en comparación con la silimarina y la metformina.

Con respecto a la concentración de glicerol en el medio (Fig. 11), los adipocitos tratados con 1 μ M de insulina liberaron 3.7 mg/dL, mientras que los tratados con dexametasona liberaron 6 mg/dL, produciendo un aumento significativo de aproximadamente el 40% ($P \leq 0.05$) en comparación con los adipocitos tratados con 1 μ M de insulina. Mientras tanto, se observó una disminución significativa en la concentración de glicerol (1,5 mg/dL, $P \leq 0,05$) en las células tratadas con silimarina; mientras, las células tratadas con metformina mostraron niveles similares de glicerol en comparación con las células incubadas con 1 μ M de insulina ($P > 0.05$). Las subfracciones SF1 y SF3 tienen un efecto dependiente de la concentración en la liberación de glicerol, es decir, una mayor concentración de las subfracciones aumenta

la liberación de glicerol, por lo que la concentración efectiva de estas dos subfracciones es de 5 µg/ml. Mientras que SF2 induce una respuesta bimodal y se observó que la concentración de 40 µg/ml mantenía la concentración de glicerol similar a los adipocitos tratados con 1 µM de insulina.

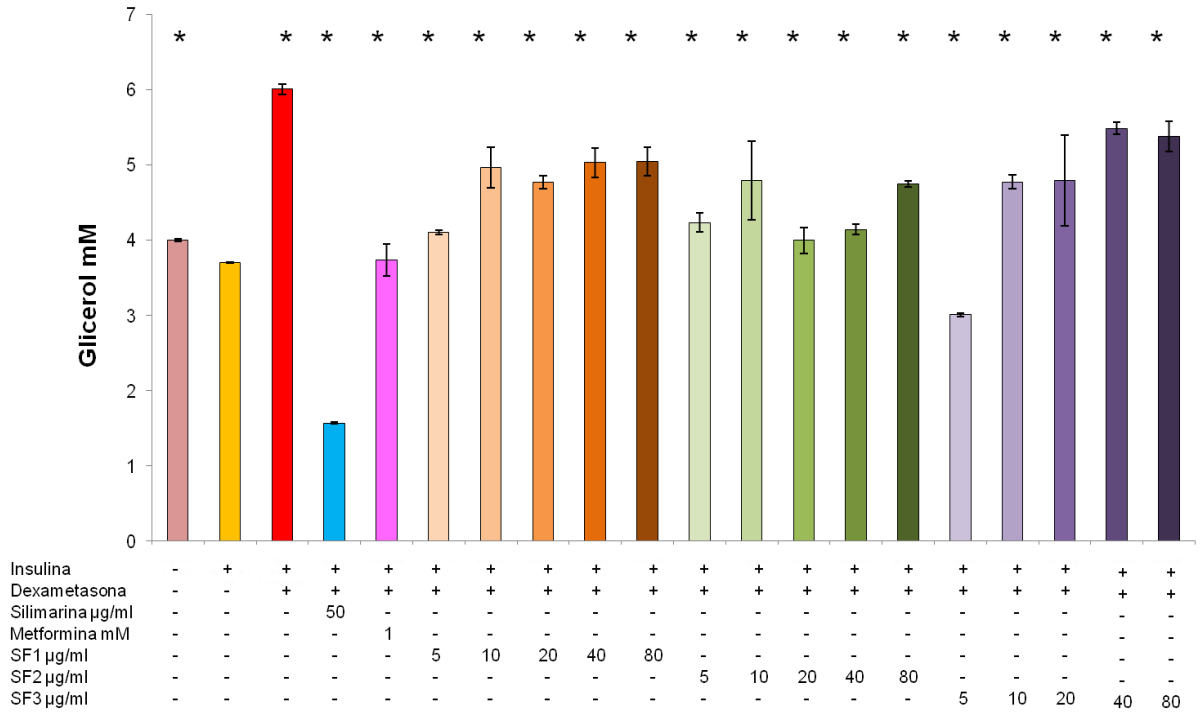


Figura 11. Efecto de las subfracciones sobre la liberación de glicerol en el medio de cultivo. Se incubaron adipocitos con 1 µM dexametasona durante 48 horas, posteriormente se agregaron los diferentes tratamientos durante 24 horas más. Los valores son representados como media±DE. Asteriscos significan diferencias estadísticas significativas (p≤0.05).

8.6 Las SF1 (40 µg/ml), SF2 (40 µg/ml) y SF3 (20 µg/ml) aumentan el consumo de 2-DG en adipocitos disfuncionales

Por otro lado se evaluó el efecto de las subfracciones de la Aq-Cs SF1, SF2 y SF3 sobre el consumo de 2-DG en adipocitos disfuncionales con dexametasona 1 µM e insulina 1 µM durante 48 horas.

Como se muestra en la Fig. 12, la dexametasona disminuyó el consumo de 2-DG en un 70% en comparación con los controles ($P \leq 0.05$). La silimarina no indujo un aumento significativo en el consumo de DG 2, pero la metformina, un fármaco utilizado para tratar la hiperglucemia en pacientes diabéticos, permitió a los adipocitos consumir un 65% más de 2-DG, produciendo una diferencia significativa con respecto a las células tratadas con dexametasona ($P \leq 0.05$). Por otro lado, SF1 y SF3 inducen el consumo de 2-DG, mostrando un efecto bimodal, en donde la respuesta máxima está en la concentración de 40 $\mu\text{g/ml}$ para los adipocitos que fueron tratados con SF1 y 20 $\mu\text{g/ml}$ para SF3. Mientras que SF2 tiene un efecto dependiente de la concentración, por lo que la concentración efectiva es de 40 $\mu\text{g/ml}$ y en donde el consumo de 2-DG es del 57%

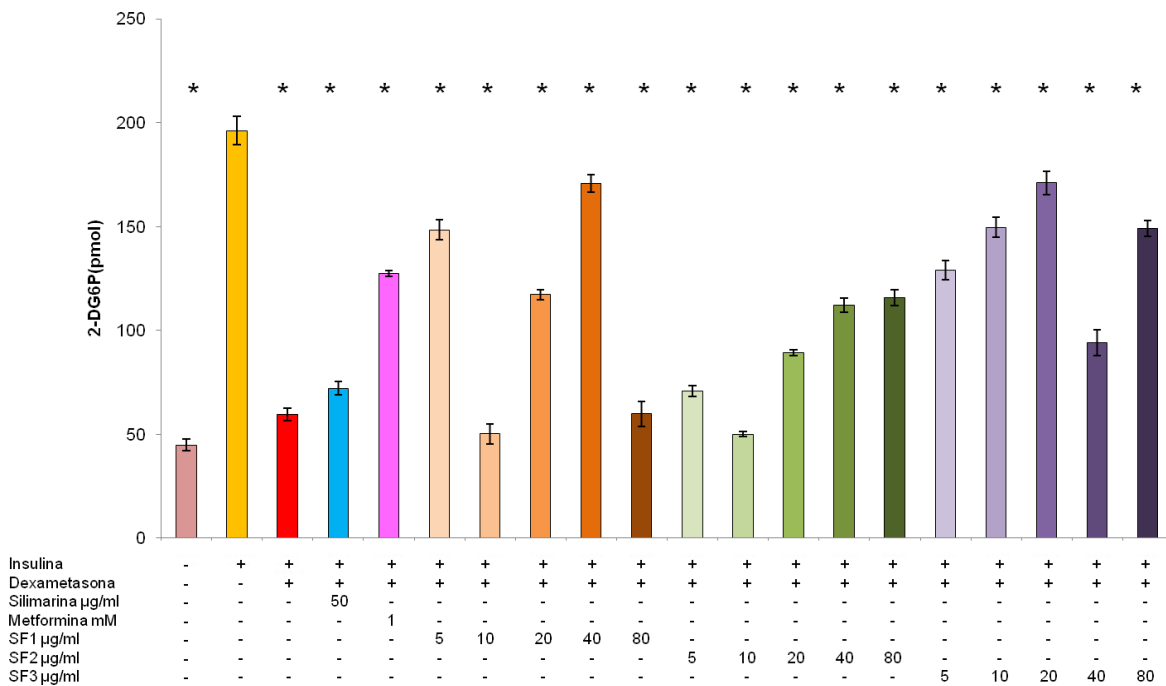


Figura 12. Efecto de las fracciones sobre el consumo de 2-DG. Se incubaron adipocitos con 1 μM dexametasona durante 48 horas, posteriormente se agregaron los diferentes tratamientos durante 24 horas más. Los valores son representados como media \pm DE. Asteriscos indican diferencias estadísticas significativas ($p \leq 0.05$).

8.7 Se obtuvieron 5 combinaciones de las 3 subfracciones que controlaron la disfunción inducida a adipocitos por la dexametasona

Con el fin de encontrar las mezclas más efectivas de las subfracciones seleccionadas para regular el consumo de 2-DG y la liberación de glicerol en adipocitos disfuncionales, se construyó un modelo de superficie de respuesta. Los resultados experimentales de dicho modelo se muestran en la Tabla 6 y las 5 combinaciones evaluadas se muestran en la Tabla 7. Los resultados indican que los valores obtenidos son comparables a los obtenidos en los controles (silimarina y metformina) ya que son superiores a 120 mg/ml para 2-DG, y entre 1 a 4 nM para de glicerol.

Las gráficas tridimensionales se obtuvieron calculando la respuesta de superficie para el consumo de 2-DG y la liberación de glicerol (Figura 13A y 13B). Tal como se muestra, las combinaciones M1, M4 y M5 tienen un aumento en el consumo de 2-DG, por lo que se encuentran en el pico de respuesta más alto en el gráfico del modelo de superficie (figura 13A y tabla 7). Los resultados obtenidos sobre la concentración de glicerol indican que, las combinaciones M1, M3, M4 y M5 mantienen esta concentración en el rango de 1-2 nM, valores que pertenecen a los niveles más bajos del gráfico del modelo de superficie (Fig. 13B y tabla 7).

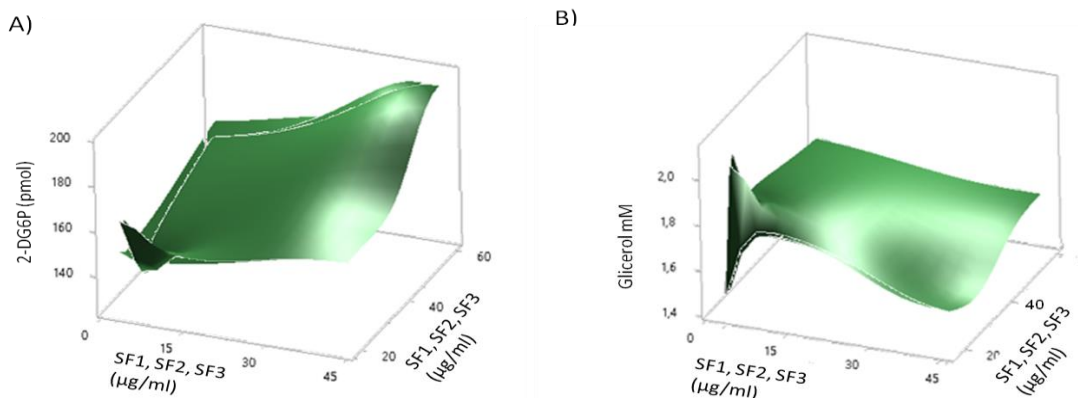


Figura 13. Gráfico de superficie de respuesta. Se construyó un modelo de superficie de respuesta para identificar las combinaciones óptimas de subfracciones a una concentración efectiva. Para esto, se agregaron combinaciones de las subfracciones efectivas a los adipocitos 3T3-L1 disfuncionales y se incubaron durante 24 h adicionales. Se evaluó el efecto de las combinaciones en el consumo de 2-DG (A) y la liberación de glicerol (B). La gráfica muestra el efecto de las combinaciones de las subfracciones efectivas para ambos parámetros.

Tabla 6. Resultados experimentales del diseño factorial fraccional

	SF1	SF2	SF3	Consumo 2-DG	Liberación de glicerol
Tratamiento	x_1	x_2	x_3	Y (pmol/dL)	Y (nM)
1*	-1	-1	-1	149.5	2.1
2*	-1	-1	0	126.5	1.8
3*	-1	-1	1	162.4	1.5
4#	0	1	-1	194.9	1.6
5#	0	1	1	160.2	1.5

*1,2,3: $x_1 = ((X_1 - 5)/2.5)$, $x_2 = ((X_2 - 40)/20)$, $x_3 = ((X_3 - 5)/2.5)$

#4,5: $x_1 = ((X_1 - 40)/20)$, $x_2 = ((X_2 - 40)/20)$, $x_3 = ((X_3 - 20)/10)$

Tabla 7. Combinaciones efectivas acorde al modelo de superficie de respuesta

Combinación	SF1	SF2	SF3
M1	2.5 µg/mL	20 µg/mL	7.5 µg/mL
M2	2.5 µg/mL	20 µg/mL	2.5 µg/mL
M3	2.5 µg/mL	20 µg/mL	5 µg/mL
M4	40 µg/mL	60 µg/mL	30 µg/mL
M5	40 µg/mL	60 µg/mL	10 µg/mL

8.8 Las combinaciones M4 y M5 disminuyen la expresión de IL-6 y de O₂⁻

Para determinar si las subfracciones exhiben actividad antiinflamatoria y antioxidante cuando se administran juntas, las cinco combinaciones obtenidas en el modelo de superficie de respuesta se evaluaron experimentalmente en condiciones de disfunción inducida por IL-1 β .

Los adipocitos tratados con IL-1 β (figura 14A), aumentaron casi el 50% en la concentración de IL-6 con respecto a los adipocitos con insulina 1 µM ($P \leq 0.05$). Las combinaciones M4 y M5 mantuvieron las concentraciones de IL-6 en el medio similar a los adipocitos tratados con insulina y silimarina ($P > 0.05$). Por lo que estas combinaciones son eficientes en su actividad anti-inflamatoria. Mientras que M1, M2 y M3 su disminución en la concentración de IL-6 fue significativa, sin embargo, no fueron tan eficientes como M4 y M5.

Para evaluar la capacidad antioxidante de las combinaciones de las subfracciones de la Aq-Cs, se determinaron los niveles de O_2^- en adipocitos disfuncionales. Como se muestran en las Figs. 14B y 15, al tratar a los adipocitos con IL-1 β se indujo un aumento de cinco veces en los niveles de O_2^- con respecto a los adipocitos tratados con insulina ($P \leq 0.05$). La silimarina, un flavonoide con actividad antioxidante, disminuyó los niveles de O_2^- en un 84.3%. La combinación M2, M4 y M5 mostraron un efecto similar al de la silimarina, mientras que M1 y M3 disminuyeron los niveles de O_2^- en 54.9% y 35%, respectivamente, comparado con los adipocitos tratados con IL-1 β , aunque estas diferencias no fueron significativas ($P > 0.05$). Estos resultados indican que los compuestos en las combinaciones mostraron una capacidad antioxidante similar a la de la silimarina.

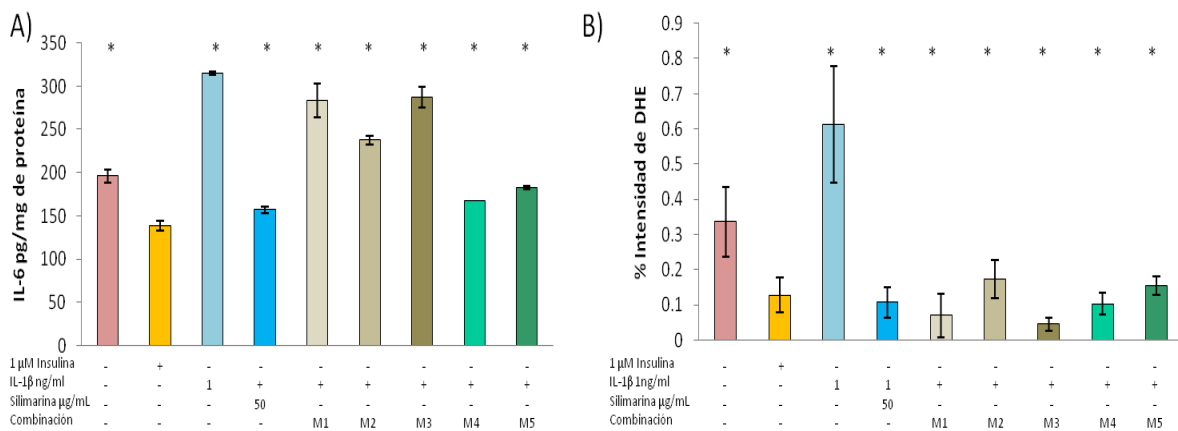


Figura 14. Efecto de las mezclas de las subfracciones en adipocitos disfuncionales. Se incubaron adipocitos con 1 ng/ml IL-1 β (a y b) durante 48 horas, terminado el tiempo de incubación se agregaron los tratamientos durante 24 horas, y se cuantificó: a) Concentración de IL-6 en el medio de cultivo; y b) Porcentaje de intensidad de DHE. Los valores son representados como media \pm DE. Asteriscos indican diferencias estadísticas significativas ($p \leq 0.05$), con respecto a las células incubadas solo con insulina.

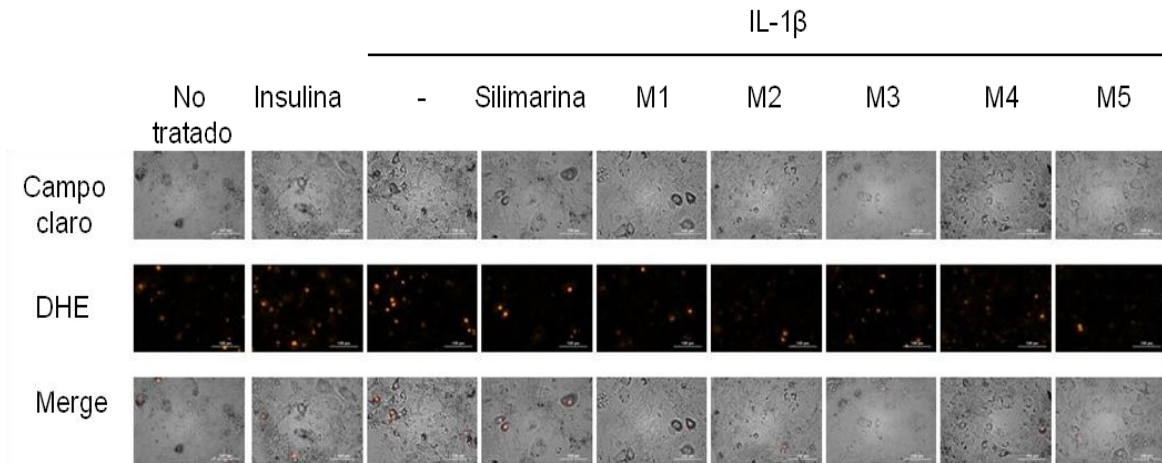


Figura 15. Efecto de las mezclas de las subfracciones en adipocitos disfuncionales. Se incubaron adipocitos con 1 ng/ml durante 48 horas, pasado el tiempo de incubación se agregaron los tratamientos y se incubaron durante 24 horas más. Y se cuantificó la expresión de DHE. Se muestran las imágenes en campo claro, campo oscuro.

8.9 Las subfracciones obtenidas a partir de la Aq-Cs contienen aminoácidos, polisacáridos y flavonoides

Con la intención de hacer un análisis cualitativo del perfil químico de las subfracciones se realizaron cromatografías de capa fina. En la figura 16 y el anexo 1 se muestran las placas cromatográficas reveladas para aminoácidos, (panel A), flavonoides (panel B) y polisacáridos (panel C). Estos resultados indicaron que las subfracciones presentan estos compuestos, siendo más marcada la presencia de compuestos que fueron revelados con ninhidrina, lo que indica que los aminoácidos pudieran ser los responsables de los efectos biológicos de las fracciones observadas en experimentos *in vitro*.

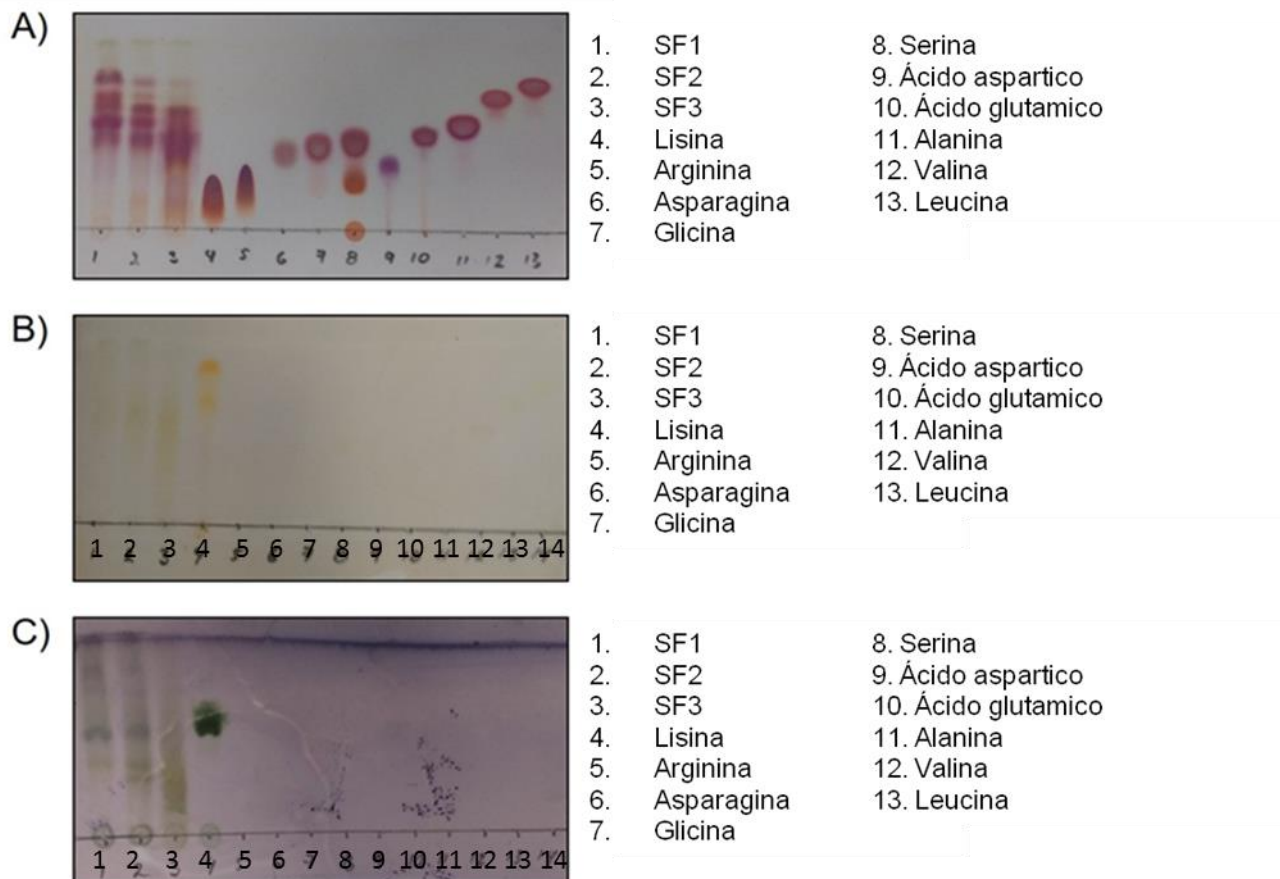


Figura 16: Análisis de los compuestos por Cromatografía en capa fina. Las subfracciones obtenidas del CsAq (F1, F2 y F3), se analizaron por cromatografía en capa fina, fase normal y el sistema n-butanol: acetona: ácido acético glacial: agua (35:35:10:20 v/v). Y fueron revelados para aminoácidos (A), con aminoetanol dimetilborato para Flavonoides (B) y 4-hidroxibenzaldehído para polisacáridos (C).

8.10 Los compuestos mayoritarios presentes en las 3 subfracciones son glicina, arginina y asparagina

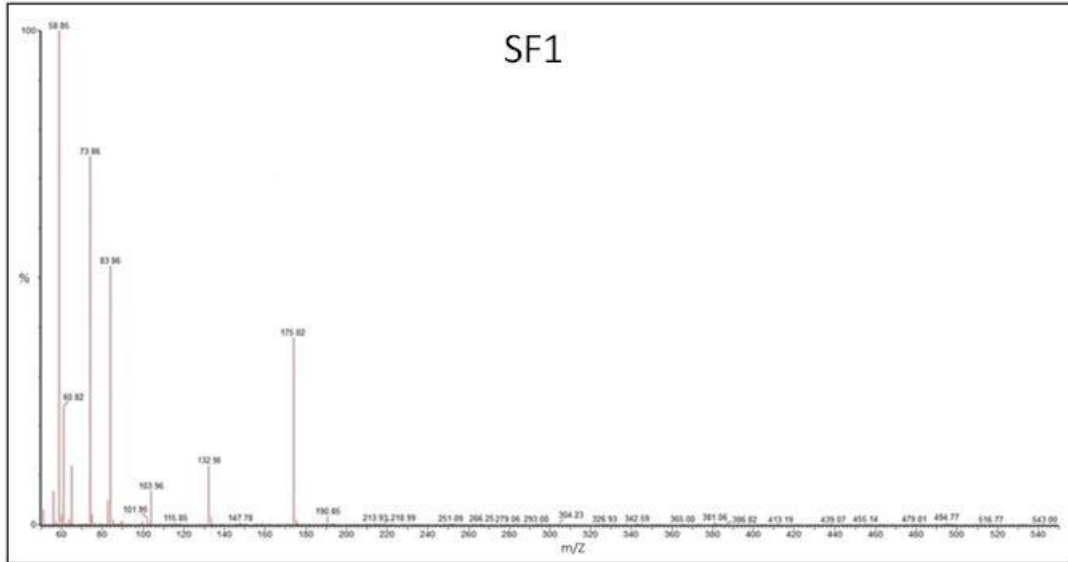
Con la intención de identificar los aminoácidos presentes en las subfracciones de la Aq-Cs que podrían subyacer a los efectos biológicos observados, se realizó un análisis de espectrometría de masas con diversos estándares de aminoácidos. Los resultados de este análisis se muestran en la Tabla 8 y figura 17. En donde se observa que la ionización positiva produjo pocos picos con un peso molecular de 58.84, 146.97 y 157.01 Da, que corresponden a los estándares de glicina, lisina y arginina, respectivamente. Por

otro lado, la ionización negativa produjo un pico con un peso molecular de 132.98 Da, que corresponde al estándar de asparagina.

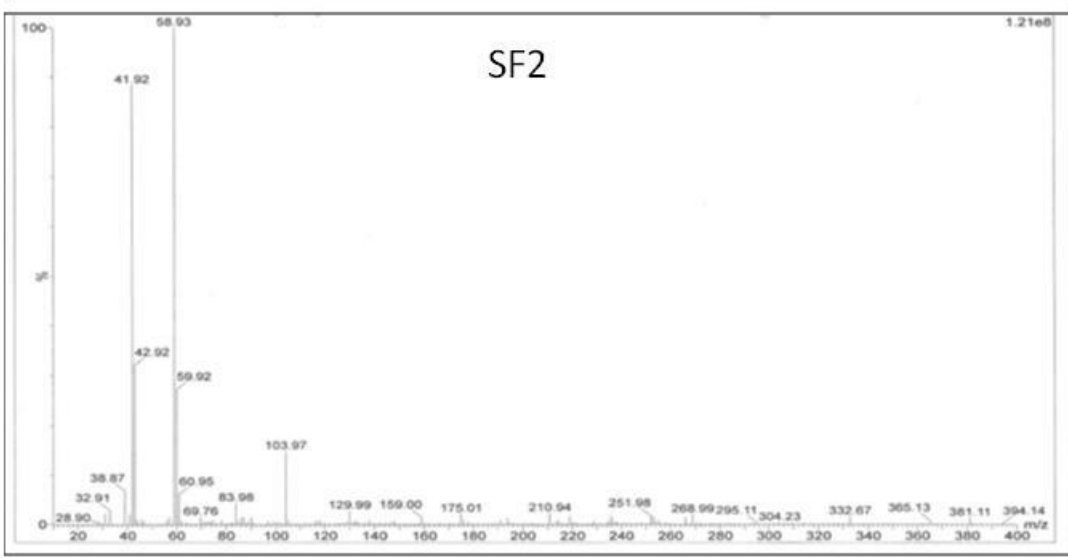
Tabla 8. Aminoácidos identificados en las subfracciones de la fracción acuosa de *Cucumis sativus* por el análisis de espectrometría de masas.

Espectrograma Ion	Pico precursor (m/Z)	Aminoácido	Fórmula molecular
Positivo	58.96	Glicina	C ₂ H ₅ NO ₂
Negativo	113.00	Asparagina	C ₄ H ₈ N ₂ O ₃
Positivo	128.93	Lisina	C ₆ H ₁₄ N ₂ O ₂
Positivo	157.97	Arginina	C ₆ H ₁₄ N ₄ O ₂

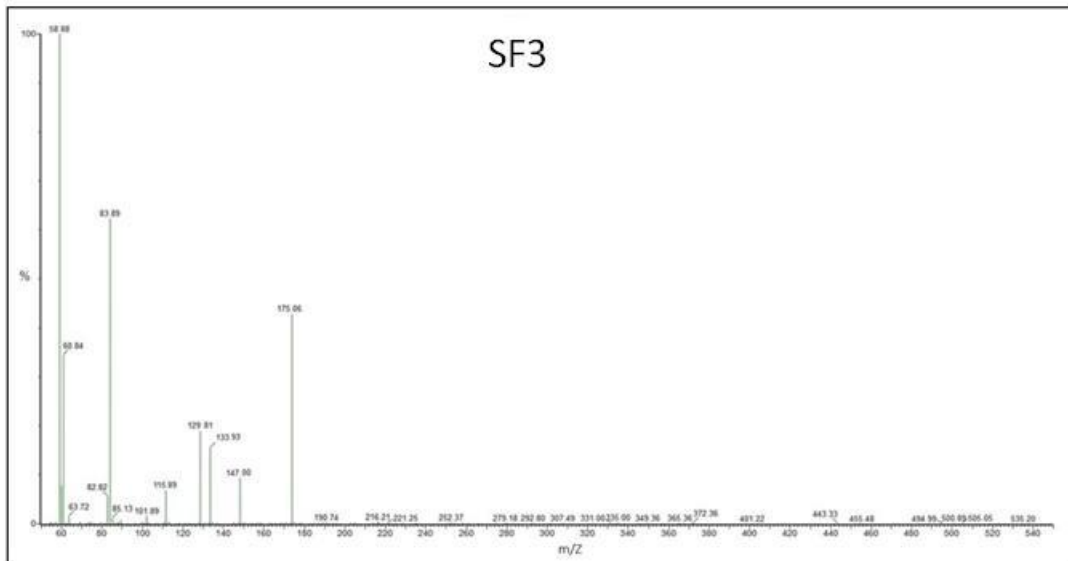
A)



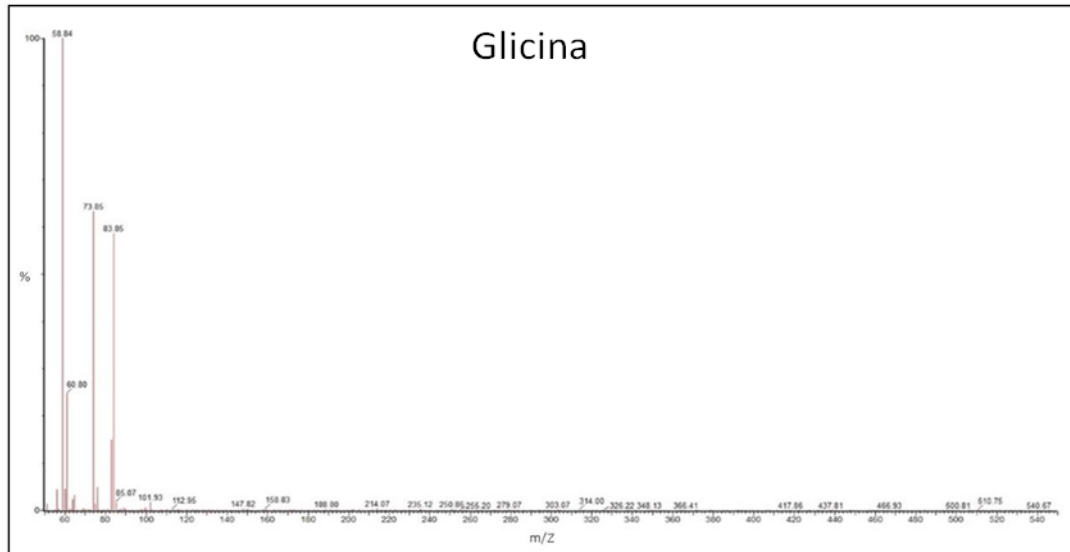
B)



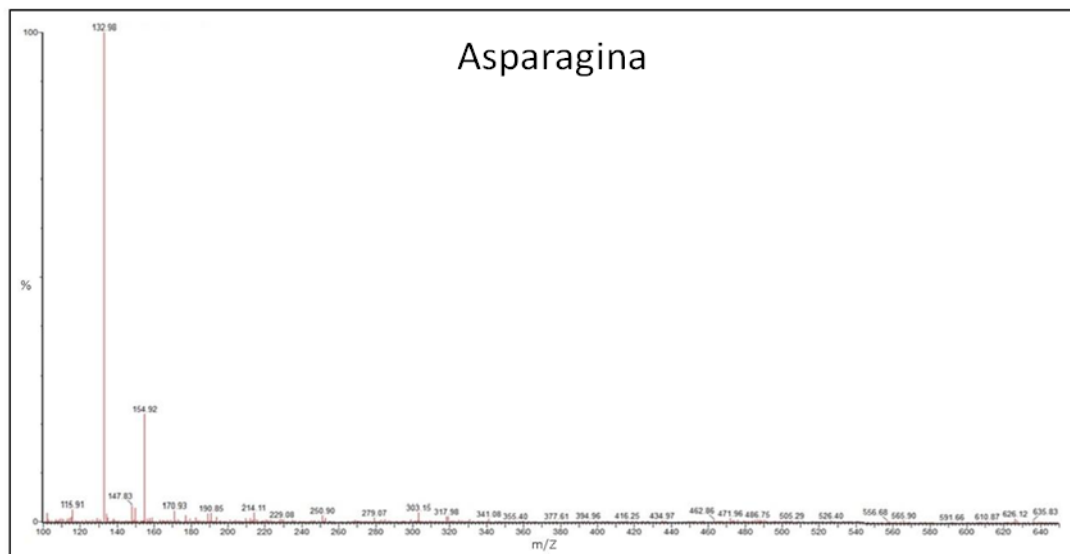
C)



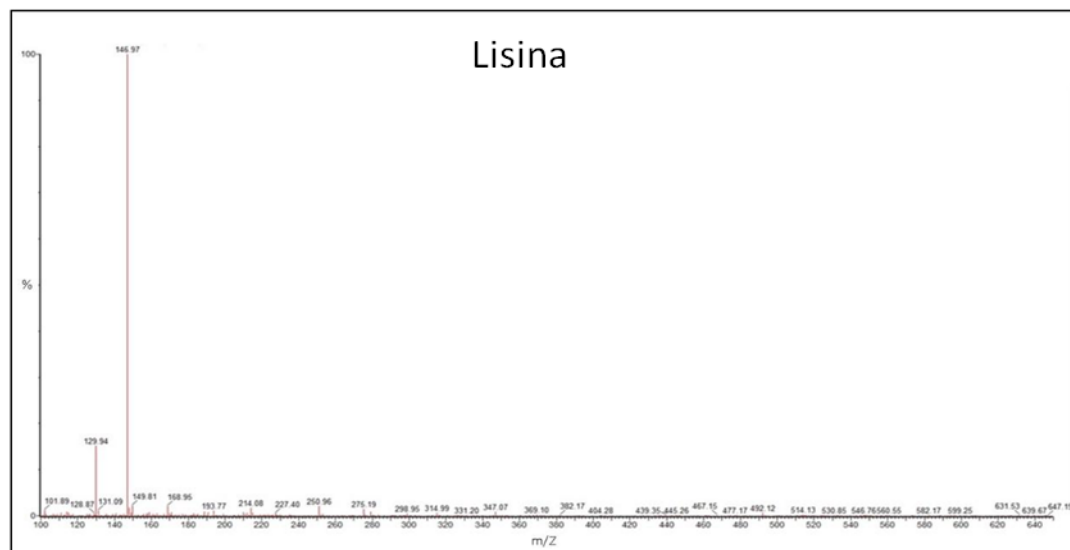
D)



E)



F)



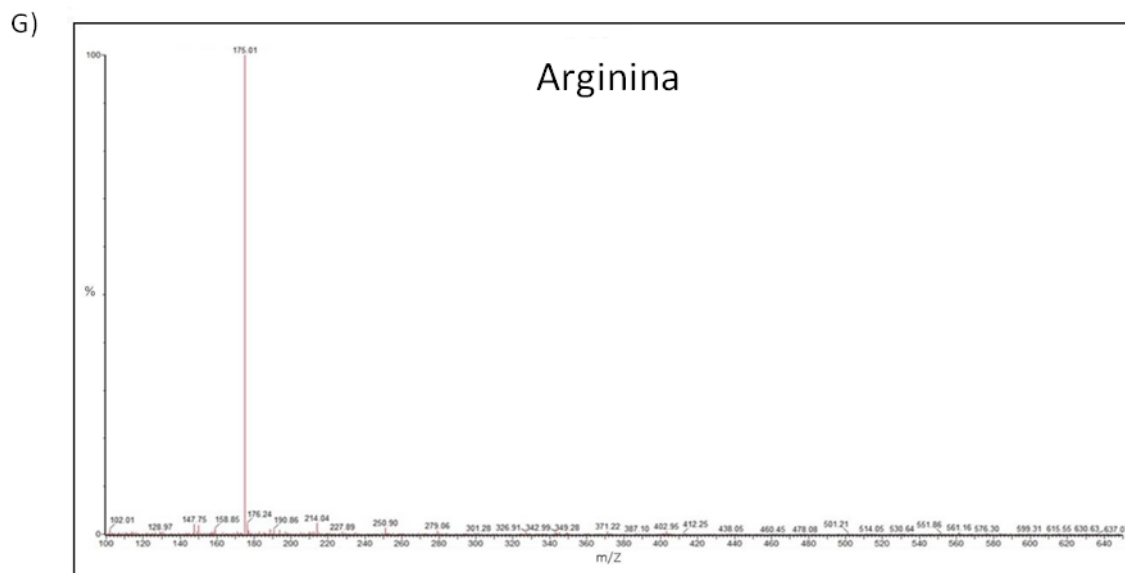


Figura 17. Espectrometría de masas. A) La SF1 contiene glicina, asparagina y arginina (picos 58.85, 132.98 y 175.02, respectivamente). B) La SF2 contiene glicina y arginina (picos 58.93 y 175.01, respectivamente). C) La SF3 contiene glicina, lisina y arginina (picos 58.88, 147.00 y 175.06, respectivamente). D) Estándar de glicina. E) Estándar de asparagina. F) Estándar de lisina. G) Estándar de arginina.

8.11 La dieta hipercalórica incrementó el peso y el Índice de Lee, parámetros que no fueron modificados por M5.

Una de las características más evidentes de la obesidad es la acumulación de masa grasa, lo que trae por consecuencia el incremento del peso y del Índice de Lee. Dicha acumulación se da por el consumo de una dieta hipercalórica y el sedentarismo, hábitos que serán difíciles de erradicar cuando el paciente se encuentre en tratamiento, por ello era importante probar la mezcla en condiciones realistas.

Ambos parámetros fueron evaluados en ratones alimentados con la DIC, la DHC o la DHC + M5 al inicio del experimento (semana 0); a las 15 semanas, lo que permitió evaluar las condiciones del ratón por efecto de la dieta y al inicio de la administración de los tratamientos y a las 25 semanas, donde lo que se evaluó fue el efecto de M5 aunado a la dieta.

En la figura 18 se muestra el peso (A) y el índice de Lee (B) a lo largo de las 25 semanas experimentales. Los resultados, indican que a las 15 semanas, los 3 grupos incrementaron ambas condiciones, pero solo los ratones alimentados con la DHC lo hicieron de manera significativa ($p < 0.05$). Esta condición se mantuvo hasta las 25 semanas, lo que indica que M5 no incide en el proceso de acumulación de masa grasa inducido por el consumo de una DHC.

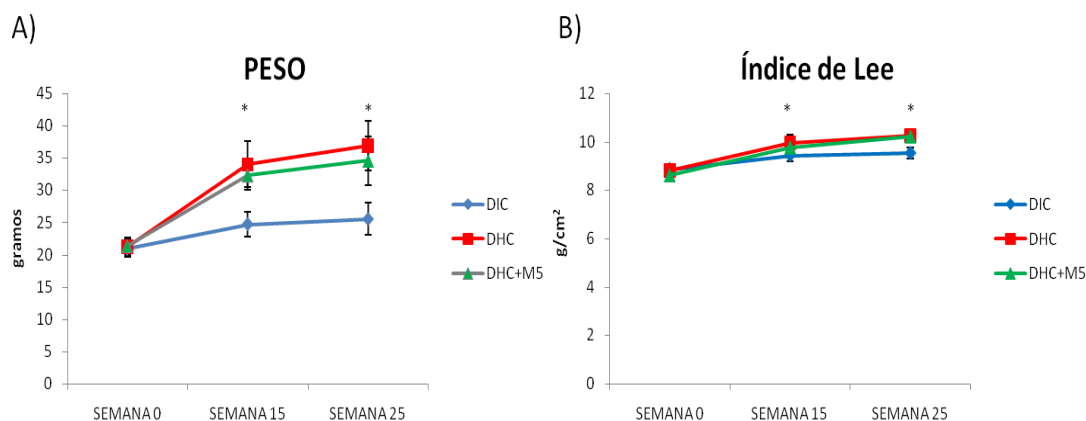


Figura 18. Peso e índice de Lee de ratones macho. Se indujo obesidad a ratones macho mediante la ingesta de una dieta hipercalórica. Las gráficas muestran las evaluaciones en la semana 0, semana 15 y semana 25 de: a) peso, y de b) índice de Lee. Los valores son representados como media \pm DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$).

8.12. La combinación M5 reguló la intolerancia a la glucosa y la resistencia a la insulina en ratones obesos

La obesidad es uno de los principales inductores de la resistencia a la insulina y de la intolerancia a la glucosa, lo que trae por consecuencia el aumento de varias moléculas, entre ellas la glucosa. Esta condición inducirá el desarrollo de *diabetes mellitus* tipo 2, que en sí misma es una parte importante del síndrome metabólico (De Ferranti 2009). La base de la intolerancia a la glucosa es la incapacidad de las células para internalizar glucosa, lo que se hace evidente a través de la prueba “curva de tolerancia a la glucosa (CTG)” en donde se administra glucosa vía oral y se espera que las células del organismo la internalicen. Con la intención de evaluar estos parámetros en los ratones tratados con M5, a las 25 semanas experimentales se les realizó una Curva de Tolerancia a la Glucosa

con los animales en ayuno y una de Tolerancia a la Insulina en condiciones postprandiales.

La comparación de las CTG de los diferentes tratamientos se muestra en la figura 19A. En ella se observa que el grupo tratado con DHC+M5 se comportan estadísticamente igual que el grupo DIC a lo largo de toda la curva, y ambos presentan concentraciones significativamente menores ($p < 0.05$) al minuto 30 y 60 (30% y el 25% respectivamente) de la misma con respecto a la de los ratones con DHC.

Estos resultados indican que la dieta hipercalórica indujo intolerancia a la glucosa que fue superada por la combinación M5, por lo que la población de ratones bajo este tratamiento no presentó diferencia estadística ($p > 0.05$) con respecto al grupo blanco.

Por otro lado, la Curva de Tolerancia a la Insulina permite evaluar si la intolerancia a la glucosa se debe a problemas en la vía de señalización de la insulina por lo que no se da la translocación de GLUT4 de manera que las células son incapaces de captar glucosa a pesar de la presencia de la insulina (Menéndez *et al*, 2009). Los resultados en el panel C de la misma figura indican que los ratones tratados con la DHC no modifican la concentración de glucosa a pesar de la administración de insulina. Mientras que los ratones tratados con la DHC+M5 no presentan diferencias significativas con respecto al grupo DIC. Ambos grupos fueron diferentes al grupo tratado con DHC a los 30, 60 y 90 minutos (34.9%, 48% y 39.1% respectivamente) mientras que para los 120 minutos experimentales todos los grupos presentaron la misma concentración de glucosa, regresando a los valores iniciales.

A partir de éstas cinéticas se calculó el área bajo la curva (ABC), de ambas curvas (figuras 19B y 19D), en donde se observa que los ratones tratados con DHC+M5 no presentan diferencias significativas con respecto a los ratones tratados con la DIC, mientras que los ratones tratados con la DHC incrementaron significativamente ($p > 0.05$) un 28% (CTG) y un 35% (CTI). Por lo que estos resultados indican que la combinación M5 contrarresta la intolerancia a la glucosa y la resistencia a la insulina inducida por la DHC.

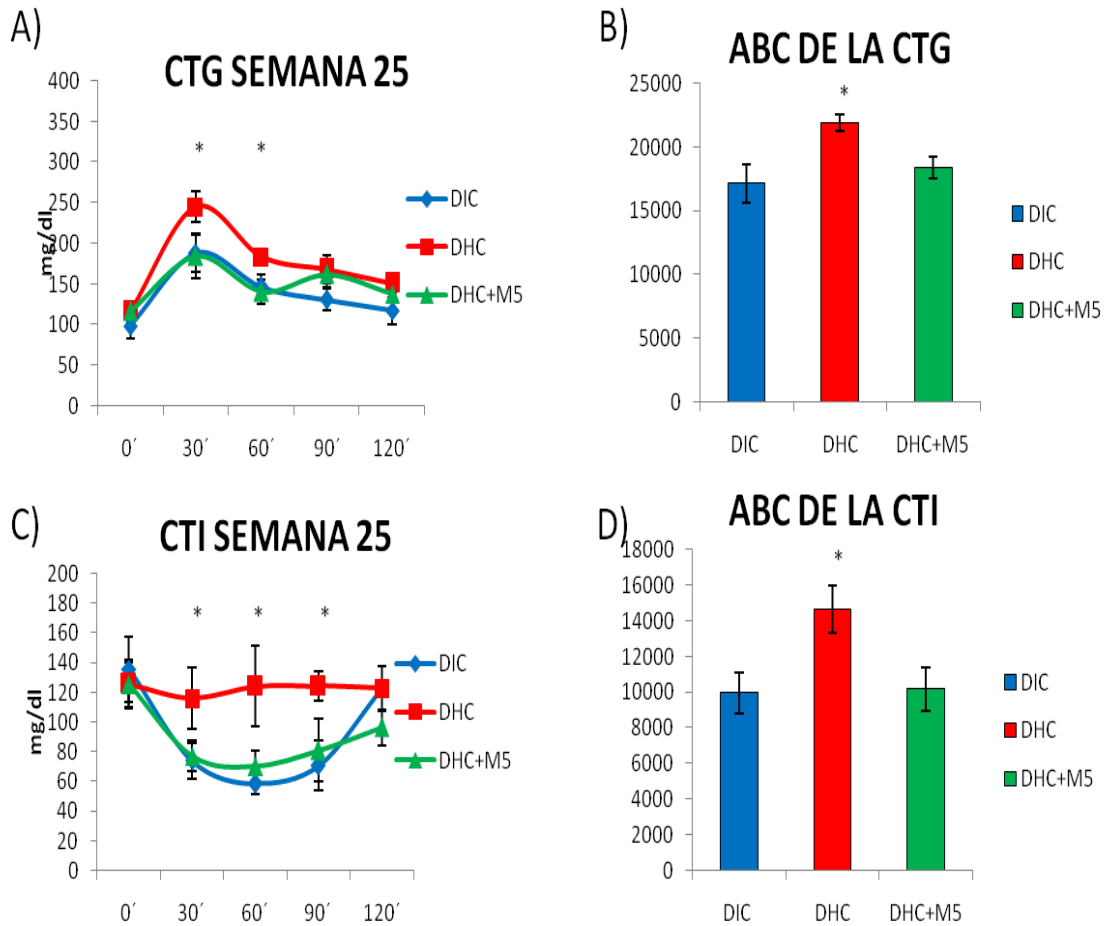


Figura 19. Evaluación de tolerancia a la glucosa y resistencia a la insulina. A la semana 25 se realizaron curvas de tolerancia a la glucosa (a) y curvas de tolerancia a la insulina, a los ratones con dieta isocalórica, ratones con DHC y ratones que recibieron la combinación M5. Por medio del método del trapecioide se calculó el ABC de las curvas realizadas (b y d). Los valores son representados como media \pm DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$), con respecto al grupo que ingirió la DIC.

8.13 M5 controla la metainflamación en ratones obesos y con resistencia a la insulina.

La metainflamación es un estado inflamatorio crónico de bajo grado que se da presente en individuos obesos y se relaciona con la producción constante de adipocinas, entre ellas IL-1 β , IL-6 y TNF- α , producidas por los adipocitos disfuncionales principalmente (Arner y Lagin 2014). En este estado se rompe el balance homeostático

que hay entre las citocinas proinflamatorias y las reguladoras como la IL-10 y el TGF- β (Yao et al., 2014).

Esta condición potencia el desarrollo de las patologías asociadas a la obesidad como la resistencia a la insulina, por ello, es importante evaluar si la combinación m5 tiene también la capacidad de controlar la metainflamación en animales que aunado a su obesidad y resistencia a la insulina continúan consumiendo de manera crónica la DHC. Para ello, a las 25 semanas experimentales, los ratones fueron sacrificados y se obtuvieron TAS, TAVE e hígado, los cuales fueron procesados para cuantificar la concentración de IL-6, IL-1 β y TNF- α (citocinas pro-inflamatorias) y de IL-10 y TGF- β (citocinas reguladoras) mediante la técnica de ELISA.

En la figura 20 se observan las concentraciones de las diferentes citocinas evaluadas. En TAS (A) se observa que los ratones con DHC en disminución significativa ($p \leq 0.05$) la concentración de IL-1 β , IL-10 y TGF- β (60.7%, 42.5% y 49.9% respectivamente) comparado con los ratones control (DIC). Mientras que el grupo tratado con M5 se mantiene baja la IL-1 β y aumenta significativamente la IL-10 y el TGF- β con respecto al grupo DHC (38%, y 21% respectivamente), incluso el TGF- β no presenta diferencias estadísticas con la concentración de esta misma citocina del grupo control. La concentración de la IL-6 y del TNF- α no varió por efecto del tratamiento. En TAVE (B) no se observan diferencias estadísticas entre los tres grupos evaluados en IL-1 β , IL-6, TNF- α , IL-10, mientras que en TGF- β se observa un aumento significativo del 20% en ratones con la DHC y lo tratados con la combinación M5. En hígado (C) también se observa la tendencia hacia un ambiente regulatorio, ya que la concentración de IL-6 disminuye significativamente ($p \leq 0.05$) (18%) con respecto a la del grupo control aunque la de las demás citocinas evaluadas no se modificó. Estos resultados indican que los animales alimentados con DHC tienden hacia un ambiente proinflamatorio que se hace evidente en TAS y en hígado, mientras que la combinación M5 lo contrarresta llevando al organismo hacia un ambiente inmunológico más regulado.

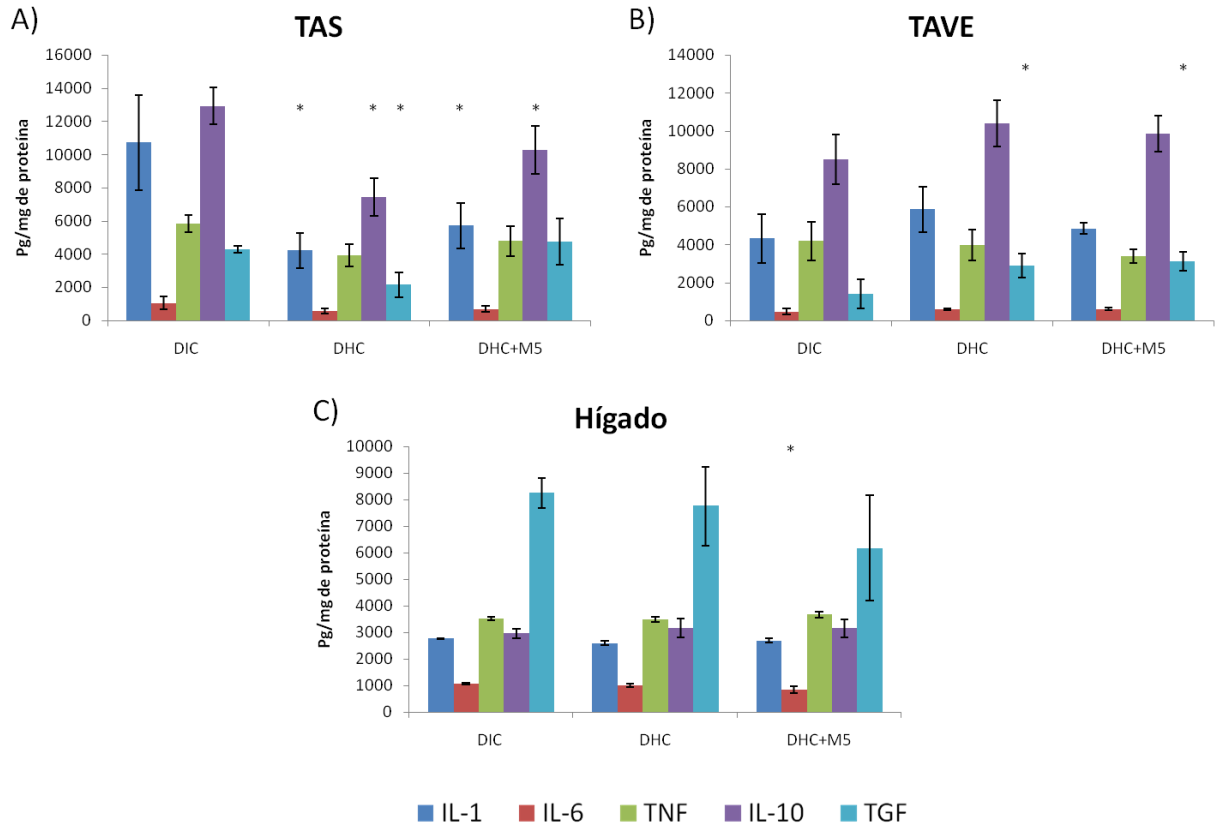


Figura 20. Cuantificación de citocinas. Las graficas muestran la concentración en pg/mg de proteína de citocinas anti-inflamatorias IL10, TGF β y citocinas pro-inflamatorias IL1 β , TNF α e IL6, en TAS (A), TAVE (B) e hígado (C). Los valores son representados como media \pm DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$).

8.14 La combinación M5 no afectó la concentración de triglicéridos ni de colesterol en suero inducidos por la dieta hipercalórica.

Otros parámetros que se observan alterados durante el desarrollo de obesidad, es la concentración sérica de triglicéridos y de colesterol. Por lo que ambos parámetros fueron cuantificados en los ratones tratados con la DIC y con la DHC en las semanas 0 y 15, así como a los ratones a los que se les administró la combinación M5 (semana 25).

Los resultados se muestran en la figura 21, en donde se observó que los ratones con la DHC incrementaron significativamente ($p \leq 0.05$) sus valores de triglicéridos en un rango de 26-30% comparado con el control de DIC en la semana 15. En la semana 25 no se observaron diferencias estadísticas significativas ($p < 0.05$) entre los tres grupos de ratones en este parámetro (Figura 21A). Mientras que, en la semana 15, la concentración de colesterol incremento en un rango de 32-49% con respecto a los ratones DIC y este

incremento se mantuvo hasta la semana 25 (Figura 21B). Por lo que, estos resultados indican que la combinación M5 no afecta estos parámetro en suero.

Con la intención de conocer si los triglicéridos y el colesterol se habían almacenado en otro órgano, se cuantifico la concentración de ambos parámetros en hígado (Figura 21, paneles C y D), este fue procesado y los resultados indicaron que durante 25 semanas aumenta un 48% ($p < 0.05$) la concentración de triglicéridos en el hígado de ratones alimentados con la DHC, mientras que los ratones alimentados con la DIC y los alimentados con la DHC pero que recibieron la M5 mantuvieron su concentración similar ($p > 0.05$). Indicando que la M5 no permitió la acumulación de triglicéridos en hígado. Con respecto a la concentración de colesterol no se observaron diferencias estadísticas significativas.

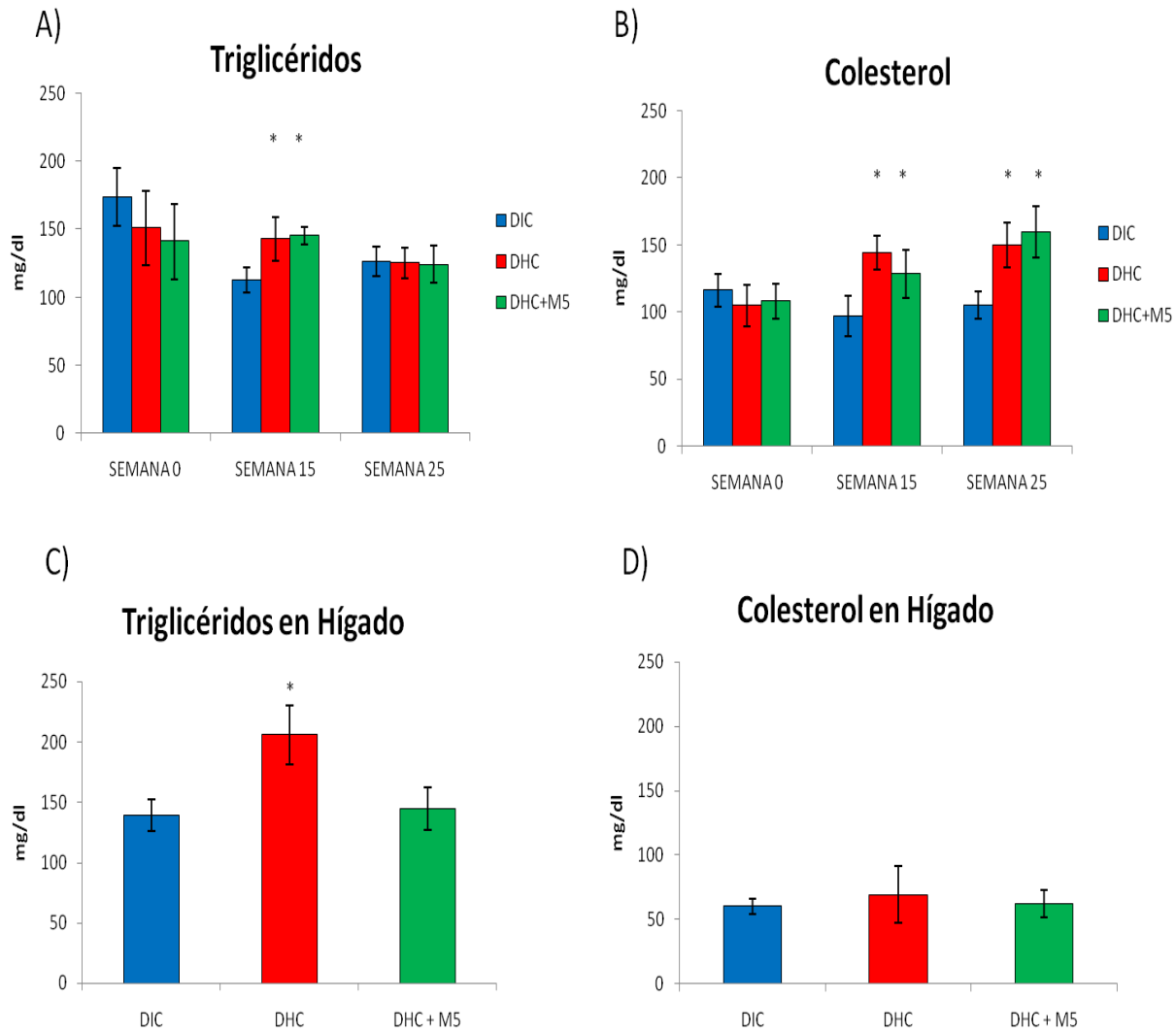


Figura 21. Concentración sérica de triglicéridos y colesterol de ratones macho. Se indujo obesidad a ratones macho mediante la ingesta de una dieta hipercalórica. Las gráficas muestran las evaluaciones en la semana 0, semana 15 y semana 25 de: A) Triglicéridos en ayunas y B) Colesterol en ayunas. A la semana 25 se cuantificó la concentración en hígado de C) Triglicéridos y D) Colesterol. Los valores son representados como media±DE. Asteriscos significan diferencias estadísticas significativas ($p \leq 0.05$).

9. Discusión

En este estudio se evaluó el efecto de las subfracciones SF1, SF2 y SF3 de la F-Aq-Cs así como sus combinaciones (M1-M5) en adipocitos 3T3-L1 disfuncionales. De las combinaciones, las M4 y M5 fueron las más eficientes, y de ellas la M5 fue probada posteriormente en ratones obesos y con resistencia a la insulina.

Dado que se ha reportado que existe relación entre la presencia de LPS en la dieta con el desarrollo de inflamación y resistencia a la insulina en el tejido adiposo en condiciones de obesidad (Hersoug *et al.*, 2018), era importante evaluar si la concentración de LPS presente en las subfracciones podría modificar los resultados, y se observó (Figura 3) que a pesar de que las fracciones si contienen LPS, las concentraciones en las que se encuentran no representaron un problema para este trabajo, ya que se ha reportado que para modificar el estado de las células se requiere de 100 ng/ml de LPS (1000 EU/ml) (Lin *et al.*, 2000; Rodríguez-Calvo *et al.*, 2009; Santos-Lira *et al.*, 2012), y en las subfracciones utilizadas se tiene 2.8 EU/ml en la SF1; 3.2 EU/ml en la SF2 y 0.2 EU/ml en la SF3, por lo que no implicó un problema para su desarrollo.

Se ha reportado que la progresión de un individuo hacia el síndrome metabólico se relaciona directamente con adipocitos disfuncionales que los lleva a la resistencia a la insulina (Fortis *et al.*, 2013). Con la intención de probar las SF-Aq-Cs primero *in vitro* antes de llevarlas *in vivo*, se indujo RI a adipocitos 3T3-L1 por medio de dexametasona, o IL-1 β . Tener dos formas de inducir la RI fue necesario porque la dexametasona, al ser antiinflamatoria no permitía evaluar en estado proinflamatorio (evaluado por la producción de IL-6) característico de los adipocitos disfuncionales (Manabe 2011; Harford *et al.*, 2011), pero permitió evaluar el consumo 2-DG y la liberación de glicerol. Por otro lado, la inducción de la RI por medio de la IL-1 β permitió evaluar tanto la capacidad antioxidante como antiinflamatoria de las fracciones.

La dexametasona altera la vía de señalización de la insulina, principalmente en la fosfatidilinositol-3-cinasa (PI3K) y en la serina/treonina proteína cinasa (Akt) por lo que evita la translocación del GLUT4 a la superficie celular induciendo así intolerancia a la glucosa (disminución de su consumo) (Jinhan *et al.*, 2015; Ottens *et al.*, 2015). En este trabajo, este efecto se midió a través del consumo de 2-DG del medio. Por otro lado, la dexametasona también aumenta la actividad de la lipasa sensible a hormonas (LSH) lo que lleva a incrementar la lipólisis en la célula, lo que lleva a una mayor liberación de glicerol y ácidos grasos libres del adipocito disfuncional hacia el medio (Xu *et al.*, 2009; Wong y Sook 2010),

Las 3 subfracciones, aunque en diferente concentración, contrarrestaron de manera eficiente la RI inducida por la dexametasona (Fig 12), lo que se puede deber a la glicina, que es la molécula que se encuentra en mayor concentración en las 3 subfracciones. Se ha reportado que la glicina: 1) actúa de manera semejante a la metformina aumentando la actividad de Akt y la expresión de GLUT4 por lo que ayuda tanto a adipocitos y hepatocitos con RI al consumo de glucosa (Chard *et al.*, 2003; Jaganjac *et al.*, 2017), 2) otro posible mecanismo para ayudar a la expresión de GLUT4 puede ser a través de la activación de PPAR γ (Alarcón *et al* 2008) ya que la glicina aumenta la secreción de adiponectina y la expresión de PPAR γ , lo cual induce sensibilidad a la insulina, 3) modula negativamente la expresión y activación de la lipoproteína lipasa (LPL) y la LSH (Reyes *et al.*, 2016) por lo que controla la lipólisis evaluada a través de la liberación de glicerol,

Otro aminoácido presente en las 3 subfracciones, aunque en diferentes concentraciones también, es la arginina. Se ha reportado que puede actuar como sustrato para la producción de óxido nítrico (ON) por la Óxido Nítrico Sintasa (NOS). Este ON, desempeña un papel clave en el metabolismo de los lípidos; ya que se ha observado que aumenta la expresión del coactivador-1 α de PPAR- γ (PGC-1 α), el cual regula la fosforilación oxidativa y la biogénesis mitocondrial, además regula la expresión de ROS (como el O $_2^-$) y, a su vez, activa la expresión de la proteína quinasa activada por AMP (AMPK), con lo que se induce la oxidación de glucosa y lípidos, no solo en tejido adiposo sino también en hígado y músculo (Bogdanski *et al.*, 2013; Wenjiang *et al.*, 2005). Otro aminoácido presente en las subfracciones fue asparagina, sin embargo no se encontraron informes con respecto a un posible efecto en el control del metabolismo.

En la RI, la inflamación y el estrés oxidante están muy relacionados porque potencian el estado de disfunción en los adipocitos (Arner y Lagin 2014). Ambas condiciones fueron inducidas por la IL-1 β porque: 1) inducen estrés en el retículo endoplásmico lo que lleva a a la producción de UCP que inducen la producción de ROS; 2) activan a la cinasa de c-jun NH2-terminal (JNK) y a IKK (inhibidor del NF- κ B) potenciando así el estado pro-inflamatorio; 3) aumentan la producción de O $_2^-$ por la mitocondria a través de la reducción catalizada por la citocromo oxidasa (Rotter *et al.*, 2003; Kagoshima *et al.*, 2003; Hsieh *et al.*, 2017). A diferencia de la dexametasona que

por su actividad antiinflamatoria no aumentó ninguno de los dos parámetros, ya que esta molécula se une y activa a sus receptores de glucocorticoides; haciendo que estos se unan a sitios específicos para la unión de NF- κ B en el ADN, reclutando cofactores y modificando la cromatina para prevenir la expresión de citocinas pro-inflamatorias (Kagoshima *et al.*, 2003); por lo que, a diferencia de la IL-1 β , esta molécula no aumentó la producción de IL-6. Así también, se ha observado que los glucocorticoides inducen indirectamente EO aumentando la peroxidación lipídica y la generación de ROS e inhibiendo las enzimas antioxidantes como la catalasa (CAT) (He *et al.*, 2017), sin embargo, en este estudio no se observaron diferencias estadísticamente significativas en la producción de O₂⁻ con respecto a las otras condiciones experimentales (Figura 8).

Los resultados indican que las tres fracciones fueron eficientes para el control de ambos parámetros porque se ha reportado que la glicina y la arginina, disminuyen la expresión del ARNm de IL-6, TNF- α , por el aumento en la secreción de adiponectina por lo que tienen efectos anti-inflamatorios (Almanza *et al.*, 2010). La glicina también actúa como un antioxidante al aumentar la biosíntesis del glutatión, que es un antioxidante protegiendo así a las células contra el EO (Angélica Ruiz-Ramírez *et al.*, 2013). La arginina también desempeña un papel clave en la regulación del EO (Wenjiang *et al.*, 2005) porque disminuye los ROS y aumenta las enzimas antioxidantes como el superóxido dismutasa (SOD), el glutatión peroxidasa (GPX) y la CAT, en adipocitos disfuncionales (Surai *et al.*, 2015). Así también ambos aminoácidos aumentan la cantidad de superóxido dismutasa de cobre/zinc (Cu/Zn-SOD), una enzima antioxidante capaz de reducir el ion superóxido a peróxido de hidrógeno (Ruíz-Ramírez *et al.*, 2013; Ren *et al.*, 2014).

Dado que las tres subfracciones fueron eficientes para controlar los diferentes aspectos evaluados de la RI, pero en diferentes concentraciones, fue necesario utilizar el Modelo de Superficie de Respuesta (Zambrano *et al.*, 2014) para predecir las mejores combinaciones de las subfracciones que indujeron una respuesta efectiva en términos del aumento en el consumo de 2-DG y una menor concentración de glicerol en el medio de cultivo (Figuras 11, 12, 13A y 13B). Al final, este modelo permitió seleccionar cinco combinaciones propuestas (M1-M5), ya que estas presentaron un control adecuado de la disfunción de los adipocitos inducida por la dexametasona. Dichas mezclas se

evaluaron en cuanto a su capacidad antioxidante y antiinflamatoria indicando que dos combinaciones (M4 y M5) (Figuras 14 y 15) exhibieron efectos antiinflamatorios (disminución en los niveles de IL-6) y antioxidantes (disminución en los niveles de O_2^-) similares a los de la silimarina, un flavonoide con actividad antioxidante y antiinflamatoria (Manna *et al.*, 1999; Peter. 2015; Shafiei-Roudbari *et al.*, 2017). Es importante hacer notar que M4 y M5 fueron las que tenían la más alta concentración de las 3 subfracciones, lo que habla de la posible eficiencia del uso del extracto de la planta para el control de las patologías asociadas al SM.

Con la intención de evaluar el efecto de la combinación M5 de las subfracciones de la Aq-Cs, se administró la M5 vía oral a ratones obesos con RI durante 10 semanas manteniendo la DHC que fue el detonador. Estos ratones, antes de administrar la combinación, mostraron características típicas de obesidad como son: 1) aumento en peso e índice de Lee (figura 18) resultado de la adipogénesis (Jeevendra *et al.*, 2008; Arner y Lagin 2014); 2) alteración en la internalización de glucosa por las células lo cual se observó en la curva de tolerancia a la glucosa (figura 19A y 19B); 3) alteración en la vía de señalización de la insulina, este efecto se observó en la realización de la curva de tolerancia a la insulina (figura 19C y 19D); y 4) una alteración en la vía metabólica de los lípidos ya que se observó un aumento en la concentración sérica de triglicéridos y colesterol (figura 21). La administración de la combinación M5 venció la intolerancia a la glucosa y la resistencia a la insulina, llevando los valores al nivel de los ratones control de DIC. Sin embargo, no se observaron efectos sobre el peso e índice de Lee, así como en la concentración sérica de triglicéridos y colesterol, este resultado concuerda con dos trabajos previos de evaluación de la fracción acuosa de *Cucumis sativus* (Méndez 2013; Maldonado 2017). Debido a que no se observaron efectos sobre triglicéridos y colesterol en suero, se evaluó la concentración de estos parámetros en hígado ya que en condiciones de obesidad se ha observado que ocurre la acumulación de grasa en otros órganos y tejidos, tales como hígado. Los resultados indicaron que la M5 controla la acumulación de triglicéridos pero no de colesterol en este órgano. Y probablemente este efecto se deba a la presencia de los aminoácidos presentes en las subfracciones.

Por otro lado, se observó que la ingesta durante 25 semanas de una DHC tiende a inducir un ambiente proinflamatorio en hígado y en TAS, lo cual se relaciona con el

proceso de metainflamación observado en obesidad (Manabe 2011; Harford et al., 2011). Y este ambiente proinflamatorio fue contrarrestado por la combinación M5 llevando al organismo hacia un ambiente inmunológico regulatorio.

De acuerdo con los resultados obtenidos en este estudio, las combinaciones M4 y M5 son las más eficientes para contrarrestar la disfunción de los adipocitos, inducida por dexametasona o por IL-1 β . Ambas combinaciones demostraron ser candidatos prometedores para un tratamiento alternativo con efectos antioxidantes y antiinflamatorios, capaces de inducir sensibilidad a la insulina y regular el metabolismo de los lípidos. Además, los efectos observados de la combinación M5 de manera in vivo probablemente se le atribuyen a los aminoácidos presentes en el extracto, ya que, como se mencionó anteriormente la glicina y arginina tienen actividad anti-hiperglucemiante y anti-inflamatoria.

10. Conclusión

Las subfracciones SF1, SF2 y SF3 de la fracción acuosa de *Cucumis sativus* y las combinaciones M4 y M5 contrarrestaron la disfunción en adipocitos.

11. Perspectivas

- Evaluar el mecanismo de acción por el cual las subfracciones llevan a cabo su actividad biológica
- Evaluar la capacidad de las mezclas efectivas sobre la adipogénesis *in vitro* e *in vivo*
- Evaluar el efecto de un extracto hidroalcohólico de *Cucumis sativus* estandarizado en la M5 para controlar la disfunción en adipocitos *in vivo*

OPEN **Effect of *Cucumis sativus* on Dysfunctional 3T3-L1 Adipocytes**

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Obesity is caused by lipid accumulation in adipose tissues inducing adipocyte dysfunction, characterized by insulin resistance, increased lipolysis, oxidative stress, and inflammation, leading to increased levels of adipokines. Herein the capacity of the subfractions (SFs) SF1, SF2, and SF3 from the *Cucumis sativus* aqueous fraction and their combinations (M) to control adipocyte dysfunction *in vitro*, in 3T3-L1 adipocytes was studied. Adipocytes, previously treated with dexamethasone or IL-1 to induce dysfunction, were incubated with different concentrations of the subfractions for 24 h. 2-deoxyglucose consumption and glycerol release were evaluated, and a surface model was constructed to determine the most effective SF concentrations to improve both parameters. Effective SF combinations were assessed in their capacity to control metabolic, pro-oxidative, and pro-inflammatory conditions. SF1, SF2 (40 µg/ml each) and SF3 (20 µg/ml) improved 2-deoxyglucose consumption by 87%, 57%, and 87%, respectively. SF1 and SF2 (5 µg/ml each) and SF3 (40 µg/ml) increased glycerol secretion by 10.6%, 18.9%, and 11.8%, respectively. Among five combinations tested, only M4 (SF1 40 µg/ml:SF2 60 µg/ml:SF3 30 µg/ml) and M5 (SF1 40 µg/ml:SF2 60 µg/ml:SF3 10 µg/ml) controlled effectively the metabolic, pro-oxidative, and proinflammatory conditions studied. Glycine, asparagine, and arginine were the main components in these SFs.

Obesity is a complex, chronic disease with multifactorial etiology. It is caused by an imbalance between energy consumption and expenditure. Excess energy is stored as fat, which accumulates mainly in adipocytes, increasing their size (hypertrophy) and number (hyperplasia)¹. Obesity incidence has increased in recent years, becoming a major health problem worldwide. In Mexico, according to the Health and Nutrition National Survey 2016, obesity affects 7 out of 10 Mexican adults (72.5% of adult population)². Obesity has been linked to dyslipidemia, hypertension, glucose intolerance, and insulin resistance (IR), leading to metabolic syndrome³. The latter increases the risk of cardiovascular diseases, diabetes mellitus type 2, cancer, and cerebrovascular stroke⁴.

Besides its function as a lipid reservoir, visceral adipose tissue is also an active endocrine organ, producing and secreting various adipokines^{5,6} which do not only modulate adipogenesis, metabolism, and adipocyte function, but also affect appetite and satiety, adipose tissue distribution, insulin secretion and sensitivity, energy release, inflammation, blood pressure, homeostasis, and endothelial function⁶. In obesity, the normal adipokine secretion by adipocytes is affected, altering their homeostasis and inducing dysfunction; lipid and glucose metabolism are first affected, inducing a local IR, which then becomes generalized^{7,8}. IR is induced by an alteration of the phosphorylation pathway of the insulin receptor substrate (IRS), in which serine and threonine residues are phosphorylated instead of the usual tyrosine residues; this results in an inhibition of the translocation of the Glucose

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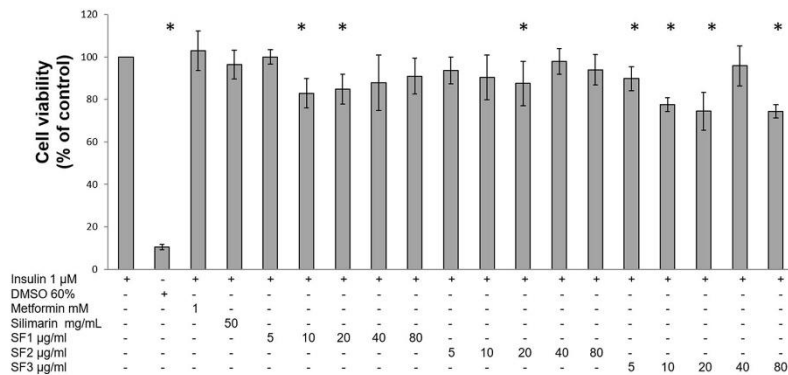


Figure 1. Effect of Aq-Cs subfractions on 3T3-L1 adipocyte cell viability. Cell viability was evaluated by the MTT assay after incubation for 24 h with the three subfractions at a concentration of 5, 10, 20, 40, or 80 μ g/mL, and with 60% DMSO. The percentage of viable cells was calculated by defining the viability of untreated cell as 100%. Cells treated with insulin 1 μ M were used as a control. Results are expressed as mean \pm SD of four independent experiments, * $P \leq 0.05$ with respect to insulin-treated cells and were analyzed by ANOVA and the post-hoc Tukey-Kramer test, $n = 6$.

transporter type 4 (GLUT4) and the induction of glucose intolerance^{9–11}. Hyperglycemia, increased levels of free fatty acids and pro-inflammatory cytokines, oxidative stress, and a constant administration of glucocorticoids like dexamethasone are known to induce IR^{9,12}.

Adipocyte dysfunction, caused by increased free fatty acid levels, induces an interaction with complexes I and II of the respiratory chain, increasing the cellular concentration of reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\bullet -}$)¹³. In adipose tissues, oxidative stress promotes glucose and lipid oxidation, producing advanced glycation end-products (AGE) and lipid peroxidation end-products (LPE), respectively, which potentiate adipocyte dysfunction and obesity-related disorders¹⁴. On the other hand, oxidative stress induces the production of IL-1 β , TNF- α and IL-6 in adipocytes¹. These cytokines induce the infiltration of macrophages with a type I phenotype and their activation in adipose tissues, which produce additional IL-1 β along with adipocyte-secreted leptin, C-reactive protein (CRP), and resistin. These events potentiate the production of TNF- α and IL-6, inducing a low-grade, chronic inflammation also known as meta-inflammation^{15,16}. In turn, these inflammation mediators can trigger oxidative stress and exacerbate adipocyte dysfunction¹⁶.

Cucumis sativus is a member of the Cucurbitaceae family; various species of this family have been used to control inflammation, oxidative stress, hyperglycemia, and dyslipidemia^{17–22}. In a recent report, the ethanolic fraction of cucumber seed extract was able to reduce total serum lipid levels in a small cohort of adult patients with mild hyperlipidemia²². In addition, the *C. sativus* aqueous fraction (Aq-Cs) was found to decrease blood glucose levels in dietary obese mice, improving insulin sensitivity and inducing a regulatory environment in epididymal visceral and subcutaneous adipose tissue (data not published). However, its effect on adipocytes is unknown. The effect of the subfractions (SFs) SF1, SF2, and SF3 of the *C. sativus* aqueous fraction and their combinations on dysfunctional 3T3-L1 adipocytes are reported herein.

IR was induced by dexamethasone and measured as the consumption of 2-deoxyglucose (2-DG) and the release of glycerol. Pro-inflammatory and pro-oxidant conditions were induced by IL-1 β and evaluated by IL-6 secretion and oxidative stress induction. Additionally, a surface response model, a mathematical/statistical tool used to analyze the interrelationship among several independent variables and their effect on one or more characteristics of a process²³, allowed us to determine the most effective subfraction combinations to control these parameters.

Results

Aq-Cs SFs did not affect adipocyte viability. Determining the effect of all SFs on adipocyte viability was a critical prerequisite to assess their capacity to control insulin resistance induced by dexamethasone and/or pro-inflammatory and pro-oxidant stress induced by IL-1 β . As shown in Fig. 1, cell viability ranged from 74.4% to 100% in cultures treated with various SF concentrations (5, 10, 20, 40, and 80 μ g/mL), with respect to metabolically healthy adipocytes (adipocytes cultured with insulin 1 μ M); in contrast, treatment with 60% DMSO caused a decrease of 89.5% in adipocyte viability with respect to healthy cells ($P \leq 0.05$).

Treatment with dexamethasone 1 μ M plus insulin 1 μ M induced 3T3-L1 adipocyte dysfunction. The administration of glucocorticoids such as dexamethasone is a well-known cause of IR^{12,24}, which is a key trait of adipocyte dysfunction. In adipocytes cultured with dexamethasone, IR is observed as a decrease in glucose consumption and/or the inability to store lipids. As shown in Fig. 2, dexamethasone 1 μ M plus insulin failed to

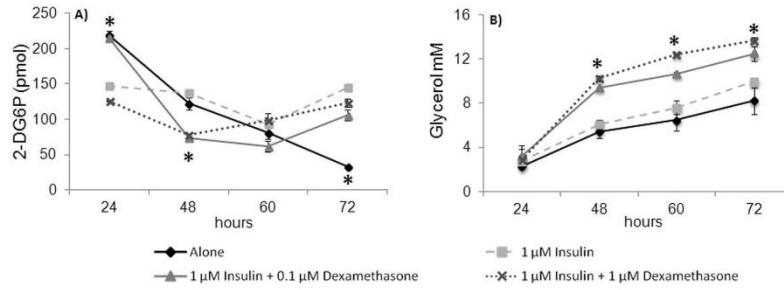


Figure 2. Effect of dexamethasone on mature 3T3-L1 adipocytes. Mature adipocytes (day 8) were cultured for 24, 48, 60, or 72 h, either alone, with insulin 1 μM with or without dexamethasone 0.1 or 1 μM. 2-DG consumption (A) and glycerol concentration in culture medium (B), were evaluated. Results are expressed as mean ± SD, *P < 0.05 with respect to cells treated with insulin 1 μM and were analyzed by ANOVA and the post-hoc Tukey-Kramer test, n = 6.

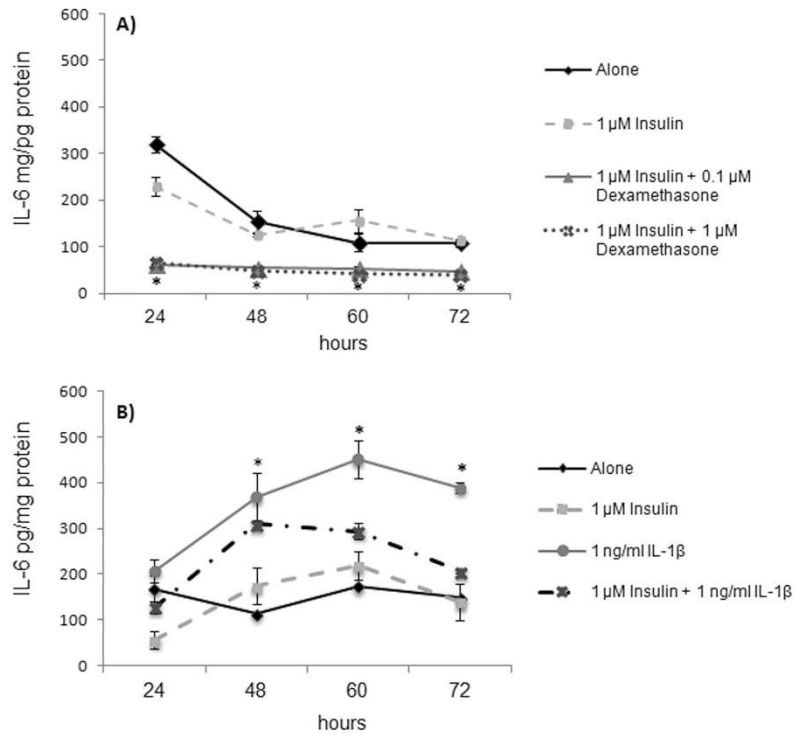


Figure 3. IL-6 (pg/mg protein) concentration in culture medium. Cells were left untreated or treated either with dexamethasone 0.1 or 1 μM (A), or with 1 ng/mL of IL-1β (B), with or without insulin. IL-6 (pg/mg protein) concentration was determined in culture medium at 24, 48, 60, and 72 h of culture. Results are expressed as mean ± SD, *P < 0.05 with respect to cells treated with insulin 1 μM and were analyzed by ANOVA and the post-hoc Tukey-Kramer test, n = 6.

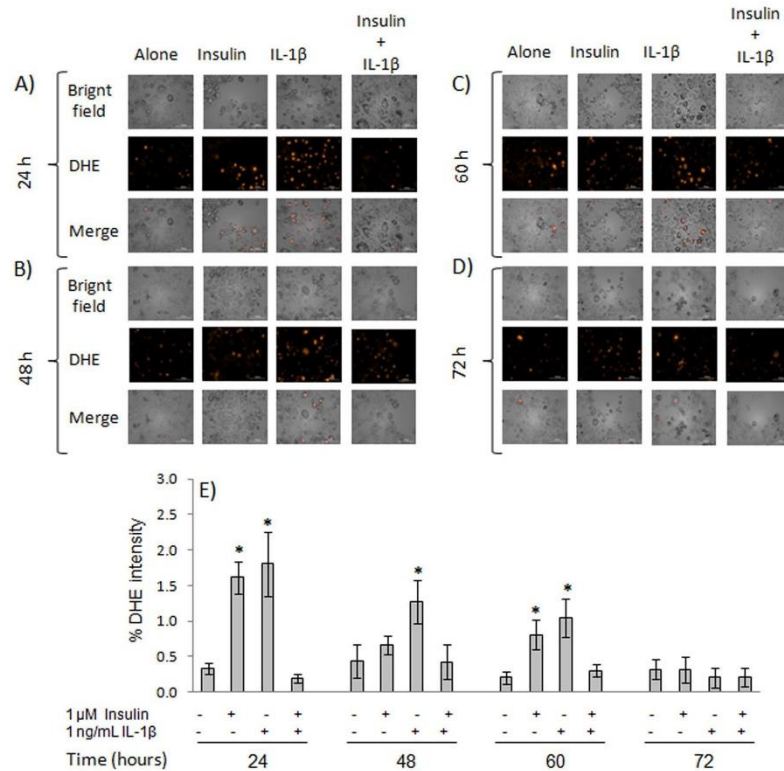


Figure 4. Superoxide induction by IL-1 β in 3T3-L1 adipocytes. Mature adipocytes (day 8) were cultured for 24, 48, 60, or 72 h, either alone, with IL-1 β , and/or insulin 1 μ M. Microphotographs were taken in a spectrofluorometer under a 20X objective at 24 (A), 48 (B), 60 (C), and 72 h (D). The percent of DHE expression was determined (E) with the software MetaMorph v.6.1. Results are expressed as mean \pm SD, * $P \leq 0.05$ with respect to cells treated with insulin 1 μ M and were analyzed by ANOVA and the post-hoc Tukey-Kramer test, $n = 6$.

after the consumption of 2-DG (a glucose analog) after 24 h of incubation, while dexamethasone 0.1 μ M plus insulin significantly decreased (14.3%, $P \leq 0.05$) 2-DG consumption with respect to cells cultured with insulin 1 μ M only (Fig. 2A). After 48 h, a decrease ($P \leq 0.05$) of 45.8% (0.1 μ M) and 43.3% (1 μ M) was observed with respect to control cultures. Finally, no significant differences were observed in 2-DG consumption with respect to control cultures after 60 and 72 h of incubation.

Glycerol concentration in the culture medium was another marker of adipocyte dysfunction. No significant differences were observed after 24 h of culture with insulin plus dexamethasone 0.1 μ M or 1 μ M ($P > 0.05$) with respect to control adipocytes (Fig. 2B). However, a significant increase ($P \leq 0.05$) of 54%, 40.1%, and 25.2% was observed in cells treated with dexamethasone 0.1 μ M plus insulin after 48, 60, and 72 h of culture, respectively. Glycerol release was increased by 67.8%, 63.4%, and 37.4% in adipocytes treated with dexamethasone 1 μ M plus insulin compared to controls after 48, 60, and 72 h, respectively.

IL-1 β induced an increase in IL-6 secretion and ROS production. Considering the capacity of dexamethasone to induce endothelial dysfunction, its capacity to induce a pro-inflammatory status and oxidative stress was assessed. However, it failed to induce a significant increase in IL-6 secretion with respect to control adipocytes at the times evaluated (Fig. 3A). A similar result was obtained in the quantification of $O_2^{\bullet-}$ (data not shown).

On the other hand, a significant, almost two-fold increase ($P < 0.05$) in IL-6 secretion with respect to healthy cells was observed when adipocytes were cultured with IL-1 β alone for 24 h (Fig. 3B). When insulin (1 μ M) was added, a nearly 3-fold increase in IL-6 levels was observed. A similar effect was observed after 48, 60, and 72 h (2.1-/1.7-, 2-/1.3-, and 2.8-/1.4-fold increases, respectively).

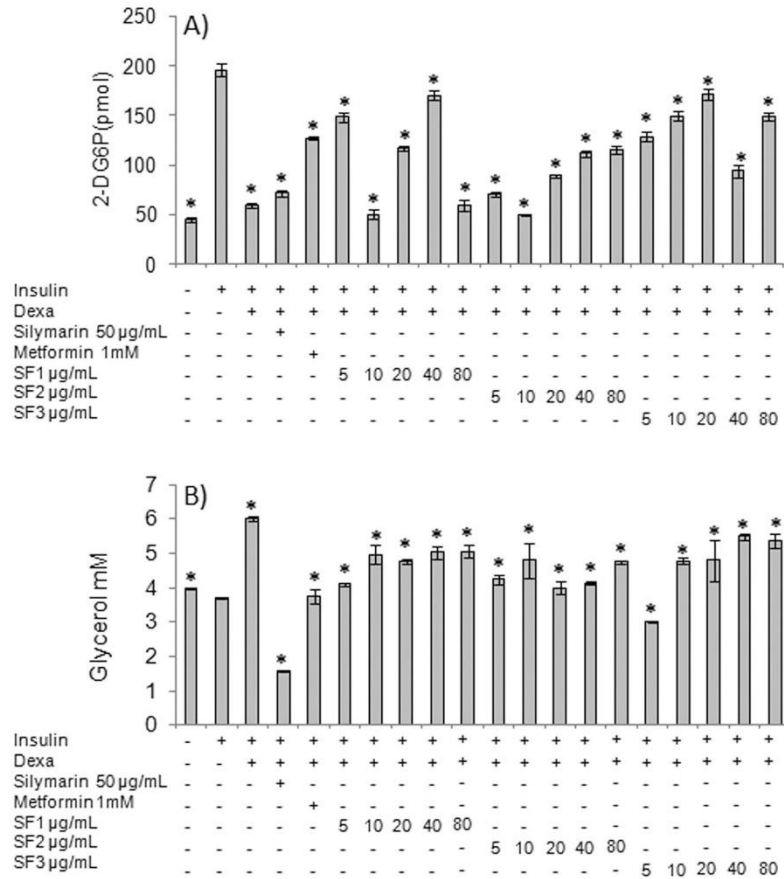


Figure 5. Effect of SF1, SF2, and SF3 Aq-Cs subfractions on 2-DG consumption and glycerol release. Mature adipocytes were cultured for 48 h with dexamethasone 1 µM plus insulin 1 µM. Then, the subfractions SF1, SF2, and SF3 were added at a concentration of 5, 10, 20, 40, or 80 µg/mL and incubated for a further 24 h period. The effect of the subfractions on 2-DG consumption (A) and glycerol concentration in culture medium (B) was evaluated. A response surface model was constructed to identify optimal combinations of subfractions at an effective concentration for 2-DG consumption (C) and glycerol release (D). Metformin and silymarin were used as controls. Results are expressed as mean ± SD, *P < 0.05 with respect to cells treated with insulin 1 µM and were analyzed by ANOVA and the post-hoc Tukey-Kramer test, n = 6.

Treatment	SF1	SF2	SF3	2-DG consumption	Glycerol release
	x_1	x_2	x_3	Y (pmol/dL)	Y (mM)
1*	-1	-1	-1	149.5	2.1
2*	-1	-1	0	126.5	1.8
3*	-1	-1	1	162.4	1.5
4*	0	1	-1	194.9	1.6
5 ²	0	1	1	160.2	1.5

Table 1. Experimental results of the factorial fractional design. *1,2,3: $x_1 = ((X_1 - 5)/2.5)$, $x_2 = ((X_2 - 40)/20)$, $x_3 = ((X_3 - 5)/2.5)$. *4,5: $x_1 = ((X_1 - 40)/20)$, $x_2 = ((X_2 - 40)/20)$, $x_3 = ((X_3 - 20)/10)$.

Combination	SF1 ($\mu\text{g/mL}$)	SF2 ($\mu\text{g/mL}$)	SF3 ($\mu\text{g/mL}$)
M1	2.5	20	7.5
M2	2.5	20	2.5
M3	2.5	20	5.0
M4	40	60	30
M5	40	60	10

Table 2. Effective combinations according to the response surface model for 2-DG consumption and glycerol release.

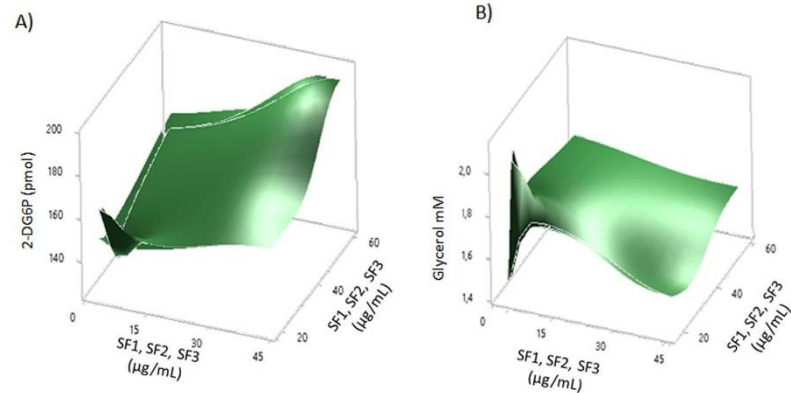


Figure 6. Response surface plot. A response surface model was constructed to identify optimal combinations of fractions at an effective concentration. For this, combinations of the effective fractions were added to dysfunctional 3T3-L1 adipocytes and incubated for an additional 24 h. The effect of the combinations on the consumption of 2-DG (A) and the release of glycerol (B) was evaluated. The plot shows the effect of combinations of the effective fractions for both parameters.

As shown in Fig. 4A,E, a significant increase ($P < 0.05$) in DHE intensity (about 50%) was observed in adipocytes treated with insulin or with IL-1 β for 24 h, indicating higher $\text{O}_2^{\bullet-}$ levels. On the other hand, treatment with IL-1 β for 48 h caused an increase of about 50% ($P < 0.05$) in DHE intensity with respect to insulin-treated cells (Fig. 4B,E). After 60 h of culture, a similar increase of about 50% ($P < 0.05$) in DHE intensity was observed in cells treated with insulin only, and also in those treated with IL-1 β (Fig. 4C,E).

These results indicate that IL-6 secretion and ROS production were increased in adipocytes treated with 1 ng/mL of IL-1 β for 48 h. Therefore, this condition was selected to evaluate the capacity of SF mixtures to control these parameters.

Aq-Cs fractions reverse adipocyte dysfunction. Adipocyte dysfunction was induced by culturing mature adipocytes with dexamethasone 1 μM and insulin 1 μM for 48 h. The effect of Aq-Cs SF1, SF2, and SF3 was evaluated by measuring glucose consumption and glycerol release compared to silymarin- and metformin-treated cells.

As shown in Fig. 5A, dexamethasone decreased 2-DG consumption by 70% compared to controls ($P \leq 0.05$). Silymarin failed to induce a significant increase in 2-DG consumption, but metformin allowed adipocytes to consume 65% more 2-DG, a significant difference with respect to dexamethasone-treated cells ($P \leq 0.05$). On the other hand, SF1 and SF3 induced 2-DG consumption, showing a bimodal effect; peak responses occurred when adipocytes were treated with 40 $\mu\text{g/mL}$ of SF1 and 20 $\mu\text{g/mL}$ of SF3. On the other hand, 2-DG consumption increased as the concentration of SF2 increased, being 40 $\mu\text{g/mL}$ and 80 $\mu\text{g/mL}$ the most effective concentrations, leading to an increase of 57% and 58.9%, respectively.

With respect to glycerol levels in culture media (Fig. 5B), adipocytes treated with insulin 1 μM only released 3.7 mM of glycerol, while those treated with dexamethasone and insulin released 6 mM, a two-fold increase ($P \leq 0.05$) compared to control cells. On the other hand, a significant decrease in glycerol concentration (1.5 mM, $P \leq 0.05$) was observed in silymarin-treated cells; metformin-treated cells showed glycerol levels similar to healthy cells ($P > 0.05$).

SF1 and SF2 increased the release of glycerol in a dose-dependent manner. The most effective concentration of both SF1 and SF3 was 5 $\mu\text{g/mL}$, while a bimodal response was observed for SF2, for which a concentration of 40 $\mu\text{g/mL}$ kept glycerol concentrations near to the levels observed in controls ($P > 0.05$).

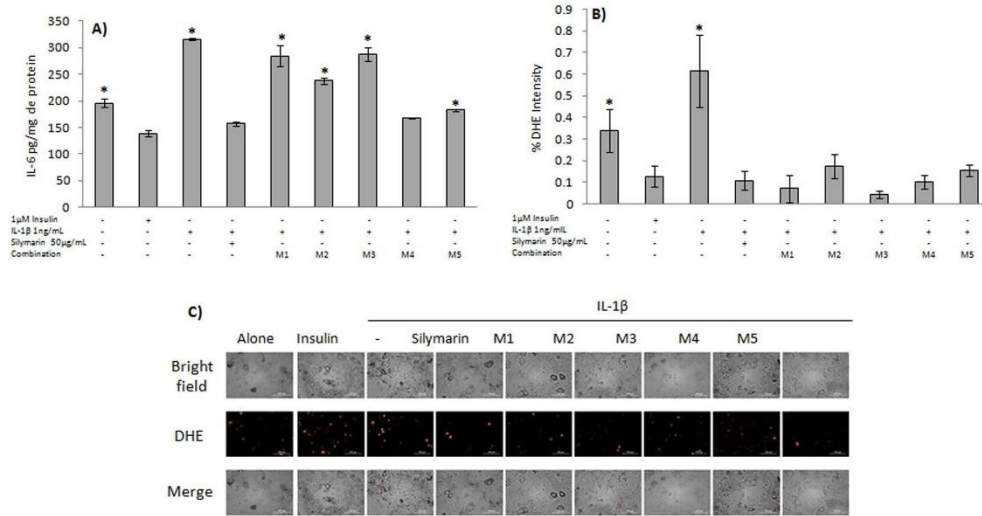


Figure 7. Effect of the combinations of effective subfractions on IL-6 production and $O_2\bullet^-$ production. Mature adipocytes were cultured for 48 h either with 1 ng/mL of IL-1 β or with insulin 1 μ M. Then, the combinations M1, M2, M3, M4, and M5 of the effective fractions were added and incubated for a further 24 h period. The effect of the combinations assayed on IL-6 concentration (A), and $O_2\bullet^-$ levels (B,C) were evaluated. Metformin and Silymarin were used as controls. Results are expressed as mean \pm SD. Microphotographs were taken in a spectrofluorometer under a 20X objective. * $P < 0.05$ with respect to cells treated with insulin 1 μ M and were analyzed by ANOVA and the post-hoc Tukey-Kramer test, $n = 6$.

Ion mode	Precursor peak (m/Z)	Amino acid	Molecular formula
Positive	58.96	Glycine	$C_2H_5NO_2$
Negative	113.00	Asparagine	$C_4H_8N_2O_3$
Positive	128.93	Lysine	$C_6H_{14}N_2O_2$
Positive	157.97	Arginine	$C_6H_{14}N_4O_2$
Positive	105.93	Serine	$C_3H_7NO_3$
Positive	73.84	Alanine	$C_3H_7NO_2$
Positive	117.96	Valine	$C_5H_{11}NO_2$
Positive	132.00	Leucine	$C_6H_{13}NO_2$
Positive	133.96	Aspartic acid	$C_4H_7NO_4$
Positive	147.96	Glutamic acid	$C_5H_9NO_4$

Table 3. Amino acids identified in the subfractions from Aq-Cs by mass spectrometry.

2-DG consumption and glycerol release response surface model. To find the most effective mixtures of the selected SFs to regulate 2-DG consumption and glycerol release, a response surface model was constructed. The results of such model are shown in Table 1, and the combinations selected are shown in Table 2. The values obtained are comparable to those observed in controls (silymarin and metformin), >120 pmol/dL for 2-DG consumption, and 1–4 mM for glycerol release.

Three-dimensional plots were obtained by calculating the surface response for 2-DG consumption and glycerol release (Fig. 6A,B). As shown, the combinations causing a peak in 2-DG consumption were M4 and M5 (Fig. 6A); these combinations kept glycerol levels in a range of 1–2 nM at the lower region of the surface model (Fig. 6B).

Combinations M4 and M5 reverse dysfunction induced by IL-1 β . To determine whether the effective subfractions exhibit anti-inflammatory and anti-oxidant activity when administered together, the five combinations obtained from the response surface model were experimentally evaluated on IL-1 β -induced dysfunctional adipocytes.

Treating adipocytes with IL-1 β induced a significant increase (by almost 50%, Fig. 7A) in IL-6 concentration with respect to healthy adipocytes ($P \leq 0.05$). The combinations M4 and M5, on the other hand, kept IL-6 concentrations close to the values observed in insulin-treated and silymarin-treated dysfunctional adipocytes ($P > 0.05$).

To evaluate the anti-oxidant capacity of the effective mixtures of Aq-Cs SFs, $O_2^{\bullet-}$ levels were determined in dysfunctional adipocyte cultures. As shown in Fig. 7B,C, adipocyte treatment with IL-1 β caused a five-fold increase in $O_2^{\bullet-}$ levels with respect to insulin-treated adipocytes ($P \leq 0.05$). Silymarin, a flavonoid with anti-oxidant activity²⁵, decreased $O_2^{\bullet-}$ production by 82.5% ($P < 0.05$). The combinations M4 and M5 showed a similar effect to that of silymarin; M1 and M3 decreased $O_2^{\bullet-}$ production by 88.6% and 92.7%, respectively, compared to IL-1 β -treated adipocytes, although no significant differences compared to silymarin nor the combinations M4 and M5 were observed ($P > 0.05$). These results indicate that the compounds in the mixtures M4 and M5 have both a significant anti-oxidant and anti-inflammatory capacity, similar to that of silymarin.

SF1, SF2, and SF3 contain amino acids. To identify the components in SFs that could be responsible for the observed biologic effects, thin-layer chromatography and mass spectrometry analyses with various amino acid standards were performed. The results (Supplementary Figs 1 and 2) indicate that amino acids are the major components of all subfractions. Positive ionization yielded few peaks with molecular weight of 58.96, 128.93, and 157.97 Da, corresponding to glycine, lysine, and arginine, respectively; negative ionization yielded a peak with molecular weight of 113.00 Da, which corresponds to asparagine (Table 3).

Discussion

Recently it was demonstrated that aqueous soluble compounds from aerial parts of *Cucumis sativus* down regulated the inflammatory and oxidative effects induced by Ang II in human endothelial cells²⁶. In this study a similar process of elimination of less polar compound (ethyl acetate fraction) was used to obtain fractions SF1, SF2 and SF3. The effect of these mixtures of metabolites obtained from the Aq-Cs fraction, as well as of their combinations (M1-M5) was assessed on 3T3-L1 adipocytes (cell line which once differentiated to adipocytes *in vitro* can be used as a model of this cell type) in which dysfunction was previously induced by culturing them in the presence of either dexamethasone or IL-1 β . Adipocyte dysfunction and IR were evaluated by the production of IL-6 and ROS like $O_2^{\bullet-}$ ¹³, as well as glucose consumption (measured as 2-DG)⁷, and the production and release of free fatty acids (measured as glycerol levels in culture medium)⁹.

Two IR inducers were used in this study, dexamethasone and IL-1 β . Dexamethasone induces IR by altering the insulin signaling pathway by inhibiting phosphatidylinositol-3-kinase (PI3K) and serine/threonine protein kinase (Akt), thus preventing the translocation of GLUT4 to the cell surface, which leads to glucose intolerance (a decrease in its consumption)^{27,28}. On the other hand, dexamethasone also increases the activity of the hormone-sensitive lipase (HSL), which results in increased cell lipolysis and therefore in an increased release of glycerol and free fatty acids by adipocytes into the medium^{29,30}. Dysfunctional adipocytes were used to evaluate the effect of the SFs and their combinations on 2-DG consumption and glycerol release (Fig. 2A,B).

In addition, being dexamethasone a synthetic glucocorticoid, acts as an anti-inflammatory, preventing the expression of IL-6 by binding and activating glucocorticoid receptors, which in turn bind specific NF- κ B-binding sites in DNA, recruiting cofactors and modifying chromatin to prevent the expression of pro-inflammatory cytokines³¹; thus, in contrast with IL-1 β , dexamethasone failed to increase IL-6 production (Fig. 3A).

On the other hand, inflammation and oxidant stress are closely related to IR, potentiating the dysfunctional status of adipocytes³². To induce both conditions, the cells were treated with IL-1 β (Figs 3B and 4A-E), which: (1) Induces stress in the endoplasmic reticulum, leading to an uncoupling protein response (UPR), which in turn leads to the production of ROS³³; (2) Increases the production of $O_2^{\bullet-}$ by mitochondria through a reduction catalyzed by cytochrome oxidase³⁴; (3) Activates the c-Jun N-terminal kinase (JNK) and IKK (an NF- κ B inhibitor), thus favoring the pro-inflammatory status and increasing the secretion of cytokines like IL-6³¹. IL-6 is regarded as a marker of adipocyte dysfunction, since it indicates a pro-inflammatory status of the cell³⁵, and it is even a marker of adipocyte damage^{35,36}. By itself, this interleukin potentiates cell dysfunction by increasing lipolysis and induces glucose intolerance in 3T3-L1 adipocytes^{36,37}, favoring IR. This is in contrast with dexamethasone, which due to its anti-inflammatory activity does not modify IL-6 nor $O_2^{\bullet-}$ levels.

The three subfractions reversed efficiently dexamethasone-induced IR at the different concentrations evaluated, with no toxic effects for the cells (Fig. 1). One of the most abundant components in all SFs was glycine (Supplementary Fig. 2); this amino acid has been reported to favor glucose consumption by glucose-intolerant adipocytes, since it helps GLUT4 to reach the cell surface and perform glucose transport. Previous reports indicate that glycine exerts these effects because it: (1) Acts in a similar manner as metformin, increasing the activity of AMP-activated protein kinase (AMPK) and therefore the expression of GLUT4^{37,38}; (2) Induces the expression of GLUT4 through PPAR γ activation and adiponectin secretion³⁹. On the other hand, glycine has also been reported to negatively modulate the expression and activation of lipoprotein lipase (LPL) and LSH⁴⁰, thus controlling lipolysis and the release of glycerol into the medium.

Another amino acid found in all three SFs is arginine, which is known to regulate lipolysis; arginine is the substrate for nitric oxide synthase (NOS) to produce nitric oxide (NO), which increases the expression of the PPAR γ coactivator 1-alpha, which in turn regulates oxidative phosphorylation. On the other hand, ON regulates the expression of ROS (like $O_2^{\bullet-}$) and AMPK, inducing glucose and lipid oxidation in adipose tissue and in liver and muscle^{41,42}. Regarding the asparagine, the other amino acid found in the SFs, no reports have been found to date on anti-hyperglycemic, anti-inflammatory, or anti-lipidemic activity of asparagine, thus further studies using this amino acid alone would be convenient to perform in order to evaluate its properties in the above pathologies of the endothelial dysfunction. Since all three SFs were efficacious (although in differing concentration levels) to control glucose intolerance and lipolysis (Fig. 5A,B), a response surface model was used to determine the best SF combinations. This model is based on the analysis of the relationship between independent variables measuring

one or more characteristics of a process⁴³, and it allowed us to select five combinations (M1-M5), which proved to induce a better control on 2-DG consumption and lower glycerol release (Table 1), being this effect even more efficient than with any SF alone (Fig. 5A,B).

These combinations were evaluated in their anti-oxidant and anti-inflammatory capacity; according to our results, two combinations (M4 and M5) exhibited anti-inflammatory effects (decreased IL-6 levels, Fig. 7A) and anti-oxidant effects (decreased O₂•⁻ levels) similar to those of silymarin, a flavonoid with well-documented anti-oxidant and anti-inflammatory activity^{44–46}.

Our results indicate that combinations M4 and M5 not only were efficient to control inflammation and oxidant stress, but also to increase the consumption of 2-DG and decrease glycerol release. Therefore, these combinations could control the IR that accompanies obesity, because it is related with the presence of oxidant stress and the secretion of IL-6^{35,36}. Glycine and arginine have been reported to decrease the expression of IL-6 and TNF- α mRNA, and therefore they have anti-inflammatory effects²⁴. Additionally, glycine acts as an anti-oxidant by increasing the synthesis of glutathione, thus protecting cells from OS⁴⁷. Arginine also plays a key role in regulating OS, decreasing the levels of ROS and increasing the levels of anti-oxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPX), and CAT in dysfunctional adipocytes²⁵. Both amino acids increase the levels of copper/zinc-superoxide dismutase (Cu/Zn-SOD), an anti-oxidant enzyme capable of reducing superoxide ion to hydrogen peroxide⁴⁸.

Considering that not all combinations were equally efficient to modulate dysfunctional adipocytes, the proportion of their constituents is relevant. Thus, it is noteworthy that M4 and M5 were the combinations with the highest concentration of the tree subfractions, indicating the possible usefulness of the plant extract to control pathologies linked to metabolic syndrome.

Conclusion

We showed that subfractions SF1, SF2, and SF3 from the *Cucumis sativus* aqueous extract and their combinations M4 and M5 are effective to control dysfunction in 3T3-L1 adipocytes, induced either by dexamethasone or IL-1 β . These two combinations proved to be promising candidates for an alternative treatment with anti-oxidant and anti-inflammatory effects, capable of inducing insulin sensitivity and regulate lipid metabolism.

Materials and Methods

Chemicals and reagents. Standards rutin, quercetin, glucose, valine, proline, glycine, arginine, leucine, asparagine, lysine, isoleucine, aspartate, and glutamate; reagents Naphthol, and aminoethanol dimethylborate, 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO), dexamethasone, insulin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), dihydroethidium (DHE), silymarin, where obtained from Sigma-Aldrich (St. Louis, MO, USA). High-glucose Dulbecco's Modified Eagle's Medium (DMEM) and calf bovine serum were purchased from ATCC (Rockville, MD, USA). Reagents 4-hydroxybenzaldehyde and ninhydrin was from Merck Millipore (Burlington, MA, USA). The pierce Coomassie Bradford Protein Assay Kit, Fetal bovine serum (FBS), Gentamicin was obtained Thermo Fisher Scientific (Waltham, MA, USA). Reagents dichloromethane, methanol, ethyl acetate, glacial acetic acid, n-butanol, acetone, acetonitrile, sulfuric acid (H₂SO₄) were purchased from J.T. Baker (Madrid).

Plant material and *Cucumis sativus* fractions. *Cucumis sativus* aerial parts, including leaves, stems, and fruits were collected from a pesticide- and fertilizer-free edible crop in Xochitepec, Morelos, Mexico, in July–August. Plant material was identified by Biol. Margarita Avilés Flores and Macrina Fuentes Mata (Herbario del Jardín Etnobotánico del Instituto Nacional de Antropología e Historia, INAH, Morelos city). Voucher specimens were stored at this site for future reference (INAH-Morelos 3001). The plant material was kept away from light at room temperature (RT) and dried in an oven at 50 °C for 36 h, following a procedure previously described²⁶. Extracts were obtained immediately after the material was completely dried. Dried plant material was ground in an electric mill to a particle size of 4 mm. This powder (1.12 kg) was extracted by a maceration process with an ethanol/water (60:40) solution at RT. The liquid extract was paper-filtered, concentrated in a rotary evaporator Laborota 4000 (Heidolph, Germany) under reduced pressure at 50 °C. The semisolid extract was finally freeze-dried to obtain 153 g of a dry green powder. The crude extract (50 g) was diluted in distilled water (500 mL) and partitioned with ethyl acetate (500 mL) in a separation funnel. The organic phase was discarded, and the volume of the aqueous fraction was reduced by low-pressure distillation to obtain a semisolid sample, which was finally freeze-dried to obtain 140 g of a green powder. This mixture (Aq-Cs, 35 g) was suspended in acetone (700 mL) for 24 h. Upon filtration, the soluble phase was concentrated and the solvent was completely removed by a distillation process under reduced pressure followed by high vacuum drying, in a rotary evaporator to obtain the subfraction SF1 (3.2 g). The precipitate was resuspended in methanol (700 mL), obtaining a solution, which was then concentrated by low pressure distillation (subfraction SF2, 4.7 g), and a precipitate (subfraction SF3, 13.3 g).

Thin-layer chromatography. Chemical profile of subfractions SF1, SF2 and SF3, was performed using a thin-layer chromatography (TLC) assay which was developed both in normal- and in reverse-phase, following a procedure previously described²⁶. Commercially available standards rutin, quercetin, glucose, valine, proline, glycine, arginine and leucine were used as references. Mixture of dichloromethane:methanol (7:3 v/v), ethyl acetate:methanol:water:glacial acetic acid (7:2:2:0.5 v/v), n-butanol:acetone:glacial acetic acid:water (35:35:10:20 and 70:70:20:10 v/v) and water:acetonitrile (7:3 v/v) were used as mobile phases. Once the plates were developed, spots were visualized with Naphthol for polysaccharides, 4-hydroxybenzaldehyde for flavonoids, aminoethanol dimethylborate for flavonoids, or ninhydrin for amino acids, following the manufacturer's instructions. The development was carried out on exposure of the plates to UV light (254 nm).

	Variable	Symbols	Levels		
			Codified ^a		
			-1	0	1
Glycerol release	SF1	X ₁	2.5 µg/mL	5 µg/mL	7.5 µg/mL
	SF2	X ₂	20 µg/mL	40 µg/mL	60 µg/mL
	SF3	X ₃	2.5 µg/mL	5 µg/mL	7.5 µg/mL
2 DG consumption	SF1	X ₁	20 µg/mL	40 µg/mL	60 µg/mL
	SF2	X ₂	20 µg/mL	40 µg/mL	60 µg/mL
	SF3	X ₃	10 µg/mL	20 µg/mL	30 µg/mL

Table 4. Experimental independent variables and levels used in this study. ^aCoded levels for the independent variables: maximum (1), intermediate (0), and minimum (-1). The amplitude of the outer levels was 50%.

ULPC-MS Analysis. To identify the bioactive compounds in SF1, SF2 and SF3, all three SFs were subjected to chromatographic analysis using an Acquity UPLC system (Waters, Milford, MA, USA), equipped with a quaternary pump and autosampler column oven. Liquid chromatography was performed at 30 °C, using an Acquity UPLC BEH 1.7 C18 column (2.1 × 10 mm × 1.7 mm i.d.). The column was eluted with a gradient system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.3 mL/min. Mobile phase gradient was set at 100% A for 2 min and subsequently ramped to 100% B (curve 6) over 14 min, followed by a 1 min at 100% B before a rapid return to 100% A, and an equilibration period of 2 min. Run-to-run time was 20 min. Volume of injection was 5 µL. Mass spectrometry analysis was performed and analyzed in a triple quadrupole TQD mass spectrometer (Waters) through an electrospray Z-spray ion source, in ESI-positive mode. Source and desolvation temperatures were 150 and 450 °C, respectively. A combination of cone voltage of 20 V and capillary voltage of 2.5 kV was used. Nitrogen was employed both as desolvation gas and cone gas. Optimal detection conditions were determined by constant infusion of standard solutions (50 µM) in solvent. MS scan was performed using argon as the collision gas. Detection conditions were determined by constant infusion of standard solutions (50 µg/mL) in acidulated water (0.05% trifluoroacetic acid). To identify the major amino acids contained in each fraction, commercial standards of glycine, arginine, asparagine, lysine, leucine, isoleucine, aspartate, and glutamate were used as chromatographic standards.

Cell culture. Murine 3T3-L1 fibroblasts were purchased from the American Type Culture Collection (ATCC CL-173; Rockville, MD, USA) and cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf bovine serum, 20 µg/mL of gentamicin and incubated at 37 °C, 5% CO₂, for 48 h. At day two post-confluence, fibroblasts were differentiated into adipocytes with dexamethasone 1.0 µM, methylisobutylxanthine 0.5 mM, and 1.0 µg/mL of insulin in DMEM plus 10% fetal bovine serum (FBS). Two days later, the medium was replaced by fresh DMEM plus 10% FBS and 1.0 µg/mL of insulin, and the cells were allowed to mature for 6 days. The cells were processed when 95% of them showed typical adipocyte traits (day 8)¹⁹.

Once adipocyte dysfunction was established as described below, five concentrations (5, 10, 20, 40, or 80 µg/mL) of the three Aq-Cs SFs (SF1, SF2, and SF3) were added to the culture and allowed to stand for 24 h. Either silymarin (50 µg/mL)^{50,51} or metformin (1 mM)^{52,53} were used as positive controls; previous reports have employed these molecules as standard drug: for silymarin as control of oxidative stress and the inflammatory condition induced by the presence of IL-1β^{45,46}. For Metformin it was used as control of glucose consumption and the accumulation of lipids in adipocytes, functions that were affected by the presence of dexamethasone²⁷⁻³⁰.

For cell viability and 2-deoxyglucose (2-DG) consumption assays, 1.5 × 10³ adipocytes/well were seeded in 96-well plates. To assess glycerol release, IL-6 concentration, and O₂^{•-} presence, 4.5 × 10⁴ cells/well were cultured in 12-well plates and further processed (see below).

MTT assay. To assess the effect of Aq-Cs SFs on cell viability⁴, mature adipocytes were incubated for 24 h in the presence of different concentrations of the three Aq-Cs SFs (5, 10, 20, 40, or 80 µg/mL) diluted in DMEM plus 10% FBS and insulin (1.0 µg/mL). Additionally, silymarin (50 µg/mL)^{50,51}, metformin (1 mM)^{52,53}, or DMSO (60%) were used as controls. After 24 h, the cells were incubated with 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at 37 °C for 4 h. After incubation, 100 µL of 10% SDS-HCl 0.01 N (1:1) were added to each well, and the plates were incubated for 2 h at 37 °C. Absorbance was measured at 570 nm in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Viable cell percentage was calculated by defining cell viability in untreated samples as 100%.

Induction of adipocyte dysfunction. Mature adipocytes were incubated for 24, 48, 60, or 72 h with dexamethasone 0.1 µM or 1 mM^{45,54}, either with or without insulin 1 µM. Some cells were cultured in the presence of 1 ng/mL of IL-1β^{31,33,34}, for 24, 48, 60, or 72 h, either with or without insulin 1 µM, as an alternate method to induce adipocyte dysfunction. Different cellular density values were employed, depending on the assay to be performed.

Deoxyglucose consumption. To select the most efficient conditions to induce adipocyte dysfunction and evaluate the effectiveness of various concentrations of Aq-Cs SFs to control metabolic dysfunction, 2-DG consumption was determined with the Uptake 2-DG kit (Sigma MAK083), following the manufacturer's instructions⁵⁵. Briefly, the cells were washed twice with phosphate buffer saline (PBS) and incubated in FBS-free DMEM for 4 h. The

cells were washed three more times and incubated for 40 min in KRPH buffer (HEPES 20 mM, KH_2PO_4 5 mM, MgSO_4 1 mM, CaCl_2 1 mM, NaCl 136 mM, and KCl 4.7 mM) plus 2% BSA. Then, the cells were stimulated with insulin 1 μM for 20 min; 2-DG 10 mM was added, mixed, and incubated for 20 min. The cells were then lysed by a cold-heat cycle, and the assay buffer plus the enzyme mixture (Sigma, Cat. No. MAK083E) (8:2) was added; the plates were incubated at 37 °C for 60 min in the dark; then, extraction buffer was added and left to stand for 5 min, and the reaction was stopped by adding Reaction Mix B (53% glutathione reductase, 42% substrate-DTNB, and 5% recycling Mix). The plates were thoroughly mixed, and absorbance was measured at 412 nm.

Glycerol release. To select the optimal conditions to induce adipocyte dysfunction and evaluate the effectiveness of various concentrations of Aq-C-S SFs to revert dysfunction, glycerol concentration was quantified in the medium using the Colorimetric Assay Glycerol Kit (Sigma, Cat. No. MAK117)⁵⁶. Briefly, 10 μL of culture medium for each treatment were transferred to 96-well plates, and 100 μL /well of the Master Reaction Mix was added; the plates were incubated for 20 min at RT, in the dark. Then, absorbance at 570 nm was measured.

IL-6 quantification in culture medium by sandwich ELISA. Adipocytes were treated for 24, 48, 60, or 72 h either with dexamethasone 1 μM or 1 ng/mL of IL-1 β . After incubation, the culture medium was harvested, and IL-6 concentration was measured by ELISA (OptEIA™ BD, 555240, San Diego, CA, USA), following the manufacturer's instructions²⁶. Briefly, flat-bottomed ELISA 96-well plates were covered with the capture antibody and incubated overnight at 4 °C in carbonate buffer 0.1 M (pH 9.6). Non-specific binding sites were blocked for 60 min at RT with 5% FBS in PBS. The samples were added, and the plates were incubated for 2 h at RT. Then, the plates were incubated with the HRP-conjugated detection anti-cytokine antibody for 60 min at RT. Tetramethylbenzidine was added as a substrate, and after 30 min of incubation at 37 °C, in the dark, the reaction was stopped with H_2SO_4 2 N. The absorbance was determined at 450 nm in a VERSAmax ELISA plate reader. IL-6 concentration was calculated as pg/mg protein based on a standard curve. Protein content was determined using the Bradford assay according to the manufacturer's instructions.

$\text{O}_2^{\cdot-}$ quantification. Superoxide anion ($\text{O}_2^{\cdot-}$) was detected using dihydroethidium (DHE), which is oxidized to ethidium⁵⁷. Briefly, adipocytes were cultured in 12-well plates and incubated either with or without 1 ng/mL of IL-1 β and/or insulin 1 μM for 24, 48, 60, or 72 h. DHE 20 μM was added five min before incubation time was completed. Then, the plates were washed three times with PBS. After adding DMEM plus 10% FBS, the cells were photographed in a Cytation 5 cell image multimodal plate lector (Biotek Instruments, Winooski, VT, USA) under a 20X objective and analyzed with the Gen 5 software. Fluorescence intensity was quantified with the MetaMorph image analysis software v.6.1 (Molecular Devices, Sunnyvale, CA, USA).

2-DG consumption and glycerol release response surface model. A 2-DG consumption and glycerol release response surface model was constructed to determine the most effective combinations of the SFs to control dysfunctional adipocytes and their concentrations²³. In the experimental design, the effect of the variables X_1 , X_2 , and X_3 , which correspond to effective concentrations of SF1, SF2, and SF3, on two response variables, Y_1 and Y_2 (2-DG consumption and glycerol release) was evaluated. The concentrations of the Aq-C-S SFs that most efficiently increased 2-DG consumption were 40 $\mu\text{g}/\text{mL}$ (SF1 and SF2) and 20 $\mu\text{g}/\text{mL}$ (SF3), and the concentrations that most efficiently decreased glycerol release were 5 $\mu\text{g}/\text{mL}$ (SF1 and SF3) and 40 $\mu\text{g}/\text{mL}$ (SF2); therefore, these concentrations were used to define the three independent variables (Table 4).

A factorial design was adjusted to 2^{3-1} quadratic polynomial models with five combinations in total (M1-M5). The variables were coded according to the following equation:

$$x_i = (X_i - X_0) / \Delta X_i \quad (1)$$

where x_i is the coded value for the independent variable; X_i is the actual value of the independent variable; X_0 is the value of the independent variable at the central point, and ΔX_i is the incremental value of the independent variable.

The predictive model for the optimal point was expressed according to the following Eq. (2):

$$Y_n = b_0 + \sum_{i=1}^3 b_i X_i + \sum_{i=1}^3 b_{ii} X_i^2 + \sum_{i < j=1}^3 b_{ij} X_i X_j \quad (2)$$

where Y_n are the response variables, b_0 is the regression coefficient, and X_i is the coded level of each independent variable. Data were analyzed by applying the regression method for the response surface, using the software Minitab® v18.1. Three levels were coded for the independent variables: maximum, intermediate, and minimum, where the amplitude of the outer levels was 50%.



Statistical analysis. All measured parameters were compiled in Excel. Differences were tested by ANOVA and the post-hoc Tukey-Kramer test. Data were analyzed with the INSTAT 3 GraphPad software by uni- and multi-varied analyses. $P \leq 0.05$ was regarded as statistically significant.

Data Availability

The material is held by the authors.

Article

Cucumis sativus Aqueous Fraction Inhibits Angiotensin II-Induced Inflammation and Oxidative Stress In Vitro

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Abstract: Inflammation and oxidative stress play major roles in endothelial dysfunction, and are key factors in the progression of cardiovascular diseases. The aim of this study was to evaluate in vitro the effect of three subfractions (SFs) from the *Cucumis sativus* aqueous fraction to reduce inflammatory factors and oxidative stress induced by angiotensin II (Ang II) in human microvascular endothelial cells-1 (HMEC-1) cells. The cells were cultured with different concentrations of Ang II and 0.08 or 10 µg/mL of SF1, SF2, or SF3, or 10 µmol of losartan as a control. IL-6 (Interleukin 6) concentration was quantified. To identify the most effective SF combinations, HMEC-1 cells were cultured as described above in the presence of four combinations of SF1 and SF3. Then, the effects of the most effective combination on the expression of adhesion molecules, the production of reactive oxygen species (ROS), and the bioavailability of nitric oxide (NO) were evaluated. Finally, a mass spectrometry analysis was performed. Both SF1 and SF3 subfractions decreased the induction of IL-6 by Ang II, and C4 (SF1 and SF3, 10 µg/mL each) was the most effective combination to inhibit the production of IL-6. Additionally, C4 prevented the expression of adhesion molecules, reduced the production of ROS, and increased the bioavailability of NO. Glycine, arginine, asparagine, lysine, and aspartic acid were the main components of both subfractions. These results demonstrate that C4 has anti-inflammatory and antioxidant effects.

Keywords: inflammation; ROS; angiotensin II; *Cucumis sativus*

1. Introduction

Inflammation and oxidative stress are two significant hallmarks of endothelial dysfunction and play a critical role in the pathogenesis of circulatory disorders such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, and chronic renal failure. These diseases are the main causes of morbidity and mortality in the world [1]. Angiotensin II (Ang II) has been implicated in the pathology of endothelial dysfunction as a source of inflammation and oxidative stress [2,3]. Ang II binds to type-1 angiotensin receptors (AT1R) on endothelial cells and promotes the activation of nicotinamide adenine dinucleotide (NADH) oxidase, which in turn increases the levels of superoxide anion ($O_2\bullet^-$), a reactive oxygen species (ROS) that decreases the bioavailability of nitric oxide (NO), which is necessary to maintain vascular tone [4,5]. Additionally, $O_2\bullet^-$ uncouples endothelial nitric oxide synthase (eNOS) and oxidizes tetrahydrobiopterin (BH4), its cofactor, leading to further production of $O_2\bullet^-$ instead of NO [6].

The presence of ROS is detected by proteins sensitive to the redox status of the cell; in turn, these proteins activate AKT (Protein kinase B), MAPKs (mitogen-activated protein kinases), and NF- κ B (Nuclear factor kappa B) [7–10], triggering inflammation and promoting the synthesis of IL-1 β , IL-6, TNF- α , C reactive protein, E-selectin, ICAM-1 (Intercellular Adhesion Molecule 1), VCAM-1 (Vascular cell adhesion protein 1), and MCP-1 (Monocyte chemoattractant protein-1), among other molecules [11–14].

Plant-derived products are attractive sources of new active ingredients for medicines, pharmacological tools, and to control various diseases improving the quality of life of patients suffering from long-lasting diseases [15]. Cucumber (*Cucumis sativus*) is a member of the Cucurbitaceae family, which includes species with therapeutic potential such as melon, squash, and pumpkin. It is a popular crop used in Indian traditional medicine since ancient times. Traditionally, this plant has been used to treat headaches and hyperlipidemia, and to prevent constipation. Seeds and the fruit have refreshing properties, soothing irritated skin and reducing swelling [16,17]. Moreover, cucumber has been reported to have antiinflammatory and antioxidant properties [17,18]. This study was aimed to evaluate the in vitro effect of the SF1, SF2, and SF3 subfractions from the *C. sativus* aqueous fraction to downregulate the inflammatory and oxidative effects induced by Ang II in human endothelial cells, and to identify the molecules potentially involved in these effects. SF1 and SF3, either alone or in combination, significantly downregulated the inflammatory and oxidative effects induced by Ang II in endothelial cells. Amino acids such as glycine, arginine, asparagine, lysine, and aspartic acid seem to be responsible for this effect.

2. Material and Methods

2.1. Plant Material and *Cucumis sativus* Subfractions

Cucumis sativus aerial parts, including fruits, were collected from an edible crop free from pesticides and fertilizers in Xochitepec, Mexico, from July through August. The plant material was kept away from light at room temperature and dried in an oven at 50 °C for 36 h. Extracts were obtained immediately once the material was completely dried. Dry plant material was ground in a Pulvex electric mill (Büchi R-114, Büchi Labortechnik, Flawil, Switzerland) until particles smaller than 4 mm were obtained. An exhaustive maceration process with ethanol/water (60:40 v/v) was performed at room temperature to obtain the hydroalcoholic extract. This extract was concentrated by reduced-pressure distillation under controlled temperature and then lyophilized. A sample of this extract (50 g) underwent bipartition with ethyl acetate/water, obtaining the aqueous fraction. This fraction was concentrated by reduced-pressure distillation under controlled temperature. Thirty-five grams of the *Cucumis sativus* aerial parts, including fruits, were collected from an edible crop free from pesticides and fertilizers in Xochitepec from July through August. The plant material was kept away from light at room temperature and dried in an oven at 50 °C for 36 h; aqueous fractions were suspended in methanol (700 mL) for 24 h; the liquid phase was filtered and concentrated in a rotary-evaporator (Laborota 4000, Heidolph, Schwabach, Germany), yielding the subfraction SF1, which was dried by lyophilization. On the other

hand, the organic phase was suspended in acetone (700 mL), yielding the subfractions SF2 (soluble phase) and SF3 (precipitate), which finally were concentrated under reduced pressure and lyophilized.

2.2. Thin-Layer Chromatography

To estimate the chemical profile of the subfractions under study, normal- and reverse-phase thin-layer chromatography (TLC) assays were performed using commercially available standards (rutin, quercetin, glucose, glycine, alanine, serine, valine, leucine, asparagine, aspartic acid, lysine, glutamic acid, and arginine). The systems dichloromethane:methanol (7:3 *v/v*), ethyl acetate:methanol:water:glacial acetic acid (7:2:2:0.5 *v/v/v/v*), and *n*-butanol:acetone:glacial acetic acid:water (35:35:10:20 and 70:70:20:10 *v/v/v/v*) were used as solvents for normal-phase TLC, while water:acetonitrile (7:3 *v/v*) was used for reverse-phase TLC. Once the plates were developed, spots were visualized with 4-hydroxybenzaldehyde (Merck, Darmstadt, Germany) and aminoethanol dimethylborate (Sigma-Aldrich, St. Louis, MO, USA) for flavonoids, and ninhydrin (Merck) for amino acids, following the supplier's directions.

2.3. Mass Spectrometry

To identify the compounds responsible for the biological activities of SF1 and SF3, a mass spectrometry analysis was performed at the facilities of the Centro de Investigaciones Biomédicas del Sur (CIBIS, Xochitepec, Mexico). A 500 µg sample was diluted in trifluoroacetic acid 0.05% to a final concentration of 50 µg/mL. Glycine, arginine, lysine, leucine, isoleucine, aspartate, and glutamate (Sigma) diluted to the same concentration were used as standards. All samples were analyzed in a triple quadrupole TQD mass spectrometer (Waters, Milford, MA, USA) coupled to an Acquity liquid chromatograph (Waters) through a combined electrospray-APCI Z-spray ion source. All samples were analyzed in positive and negative ion modes. Finally, amino acids were identified in the samples by mass spectra comparison with the standards.

2.4. Cell Culture

Human microvascular endothelial cells-1 (HMEC-1) were provided by Aida Castillo from the Department of Physiology, Biophysics and Neurosciences, CINVESTAV-IPN, México City. The cells were cultured in MCDB-131 medium supplemented with fetal serum bovine (FBS) %, L-glutamine 10 mM, 100 U/mL of penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 10 ng/mL of endothelial growth factor, and 1 µg/mL of hydrocortisone (Sigma) at 37 °C under a CO₂ atmosphere (5%). All experiments were performed using passages 3 to 8. The cells were cultured in 24-well plates to a density of 5×10^5 cells/well in 500 µL of medium supplemented with Ang II at a concentration of 0, 8, 40, 200, 1000, or 5000 nM and 0.08 or 10 µg/mL of SF1, SF2, or SF3, either for 12 h to evaluate the antiinflammatory effect, or for 6 h to evaluate the antioxidant profile of the subfractions. As a control, HMEC-1 cells were treated with 10 µmol of losartan [19] instead of the subfractions. In some experiments, the subfractions SF1 and SF3 were combined at a concentration of 0.08 µg/mL each (C1), SF1 0.08 µg/mL and SF3 10 µg/mL (C2), SF1 10 µg/mL and SF3 0.08 µg/mL (C3), and SF1 and SF3 (10 µg/mL each) (C4). For ROS and NO determination, the cells were stimulated with Ang II 5000 nM.

2.5. Immunocytochemistry

HMEC-1 cells were cultured on poly-L-lysine-coated coverslips in 24-well plates under the conditions described above. By the end of culture time, the cells were washed three times with PBS (Phosphate Buffer solution) (NaCl 140 mM, KCl 2 mM, and K₂HPO₄ 1.15 mM) and fixed in acetone for 15 min. Endogenous peroxidase was blocked with H₂O₂ 30% for 10 min. Then, the cells were incubated with PBS-BSA (Bovine Serum Albumin) 0.1%-Tween 20 0.05% (Sigma) to block unspecific binding sites. The cells were incubated with primary mouse anti-human anti-ICAM-1 and anti-E-selectin antibodies (1:100 and 1:50, respectively) (eBioscience, Waltham, MA, USA) overnight at 4 °C. The cells were washed with PBS and incubated with goat anti-mouse biotinylated IgG antibody (1:500) (Santa Cruz

Biotechnology, Dallas, TX, USA) for 30 min at 37 °C, followed by incubation with HRP (horseradish peroxidase)-streptavidin (eBioscience). The chromogenic substrate 3,3'-diaminobenzidine (BioCare Medical, Pacheco, CA, USA) was added, and 5 min later the reaction was stopped by washing the slides with water. Then, the cells were counterstained with hematoxylin. Images were obtained with an ECLIPSE 80i microscope (Nikon, Tokyo, Japan).

2.6. Western Blot

HMEC-1 cells were washed with PBS and lysed by incubating with ice-cold RIPA (Radioimmunoprecipitation assay) lysis buffer (Tris 20 mM, pH 7.4; NaCl 150 mM, EDTA (Ethylenediaminetetraacetic acid) 1 mM, pH 7.4; Triton X-100 0.5%; SDS (Sodium dodecyl sulfate) 0.1%, sodium deoxycholate 0.5%), using a cocktail of phosphatase and protease inhibitors (sodium fluoride (NaF) 25 mM; sodium pyrophosphate (NaPPi) 1 mM; sodium vanadate (NaVPO₄) 1 mM; phenylmethylsulfonyl fluoride (PMSF) 1 mM; pepstatin A 0.1 mg/mL; leupeptin 0.1 mg/mL; antipain 0.1 mg/mL; aprotinin 0.1 mg/mL) (Sigma) for 15 min. The lysates were scraped off the plates and centrifuged at 13,000 rpm for 10 min at 4 °C. Proteins in the supernatant were quantified by the Lowry method. Twenty micrograms of protein were denatured by boiling for 5 min in sample buffer, separated by SDS-PAGE (polyacrylamide gel electrophoresis) in acrylamide 10% gels and electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Merck) using transfer buffer (Tris 25 mM, pH 8.5; glycine 193 mM; methanol 20%). Membranes were blocked for 1 h in blocking buffer TBS-T (Tris-buffered saline containing Tween-20) 0.1% with BSA, 2%. Thereafter, the membranes were incubated overnight with primary anti-E-selectin (Invitrogen) or anti- α -actin (Biolegend, San Diego, CA, USA) antibodies diluted 1:1000 or 1:3000, respectively, in blocking buffer. After washing three times for 5 min with TBS-T, the blots were incubated with goat anti-mouse (Thermo Scientific, Waltham, MA, USA) or anti-rabbit (Abcam, Cambridge, UK) antibodies, diluted 1:2500. Antibody binding was detected with the SuperSignal West Dura Extended Duration Substrate solution (Thermo Scientific). The bands were analyzed using the ImageJ software (National Institute of Health, Bethesda, MD, USA) and the anti-actin signal was used for data normalization.

2.7. ROS Quantification

Intracellular ROS levels were quantified by dihydroethidium (DHE), which is oxidized by superoxide to yield ethidium; the latter binds nuclear DNA and emits a red fluorescence (535 nm Ex; 610 nm Em). The substrates NADH (1 mM), succinate (5 mM), L-arginine (1 mM), and xanthine (1 mM) were added to identify ROS sources, along with the inhibitors: DPI (Diphenylethiodonium) (0.1 mM) to inhibit NADPH (nicotinamide adenine dinucleotide phosphate) oxidase; antimycin (0.05 mM) for mitochondrial complex II; L-NAME (N(ω)-nitro-L-arginine methyl ester) (1 mM) for eNOS; and allopurinol (0.02 mM) for xanthine oxidase.

Fifteen minutes later, the cells were incubated with DHE 20 μ M and washed three times with warm PBS. Then, the cells were placed in fresh medium, analyzed, and photographed in a Cytation 5 cell imager multimodal plate reader (Biotek Instruments, Winooski, VT, USA), using the Gen 5 software (Biotek Instruments) under a 20 \times objective. Finally, the intensity of DHE emission was quantified with Metamorph v. 6.1 (Molecular Devices, San Jose, CA, USA).

2.8. ELISA

Cell culture medium was collected after a 12-h culture, and IL-6 concentration was measured by ELISA (OptEIA kit BD-Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's directions. Briefly, 96-well plates were sensitized with the capture antibody overnight at 4 °C. The plates were blocked with PBS-FBS 10% for 1 h at room temperature. Then, either medium or the IL-6 standard was added and incubated at room temperature for 2 h, followed by incubation for 1 h at room temperature with the horseradish peroxidase-coupled detection antibody. The chromogenic substrate tetramethylbenzidine (TMB) (Invitrogen) was added, and the reaction was stopped 30 min later with

H₂SO₄ 2N. Absorbance was determined at 450 nm at 37 °C using a VERSAmax microplate reader (Molecular Devices, San Jose, CA, USA). IL-6 concentration was calculated according to a standard curve.

The results obtained were used to plot a concentration–response curve and to determine the maximal effect (E_{max}) and effective 50% concentration (EC_{50}) values for each subfraction.

2.9. NO Quantification

NO levels were measured as nitrite/nitrate (final products of NO metabolism) by the Griess reaction [20]. A 100- μ L aliquot of the medium was placed in 96-well plates and incubated with 100 μ L of Griess reagent ((1-naphthyl)-ethylenediamine 0.1% and 1% sulfanilamide in phosphoric acid 2.5%) for 30 min at room temperature. Absorbance was measured at 540 nm in a VERSAmax microplate reader (Molecular Devices). The amount of NO in each sample was determined by a sodium nitrite standard curve.

2.10. Statistical Analysis

Data are reported as mean \pm SD (standard deviation). Significant differences among conditions were determined by ANOVA and the post-hoc Tukey test, with a significance value of $p < 0.05$.

3. Results

3.1. SF1 and SF3 Controlled IL-6 Production

As seen in Figure 1, the production and release of IL-6 by endothelial cells exposed to the Ang II stimulus showed a concentration-dependent behavior, $E_{max} = 31.4$ pg/mg and $EC_{50} = 14.9$ nM. Treatment with losartan, a pharmacological antagonist (a competitive inhibitor of AT1R), decreased the values of the pharmacological constants of Ang II, $E_{max} = 19.7$ pg/mg and $EC_{50} = 5.8$ nM. On the other hand, both concentrations (0.08 and 10 μ g/mL) of SF1 and SF3 antagonized the effect of Ang II: $E_{max} = 21.3$ pg/mg and $EC_{50} = 5.8$ nM (panel A); $E_{max} = 20.4$ pg/mg and $EC_{50} = 4.3$ nM (panel D); $E_{max} = 19.2$ pg/mg and $EC_{50} = 3.5$ nM (panel C); and $E_{max} = 17.4$ pg/mg and $EC_{50} = 4.9$ nM (panel F). Finally, SF2 failed to control IL-6 production in any of the assessed concentrations: $E_{max} = 24.7$ pg/mg and $EC_{50} = 4.4$ nM (panel B), and $E_{max} = 27.0$ pg/mg and $EC_{50} = 4.0$ nM (panel E).

3.2. C4 Is the Best Combination to Control IL-6 Production

To identify the most effective combination of the subfractions to control the proinflammatory status, HMEC-1 cells were cultured with Ang II and four different combinations of SF1 and SF3. Concentration–response curves showed an $E_{max} = 33.0$ pg/mg and $EC_{50} = 16.9$ nM for Ang II; losartan antagonized the effect of Ang II, $E_{max} = 19.2$ pg/mg and $EC_{50} = 4.4$ nM (Figure 2). All four combinations decreased the value of these pharmacological constants; nevertheless, C4 was the most effective: $E_{max} = 23.6$ pg/mg and $EC_{50} = 4.3$ nM (panel A); $E_{max} = 18.1$ pg/mg and $EC_{50} = 3.1$ nM (panel B); $E_{max} = 20.5$ pg/mg and $EC_{50} = 3.9$ nM (panel C); and $E_{max} = 16.7$ pg/mg and $EC_{50} = 2.9$ nM (panel D).

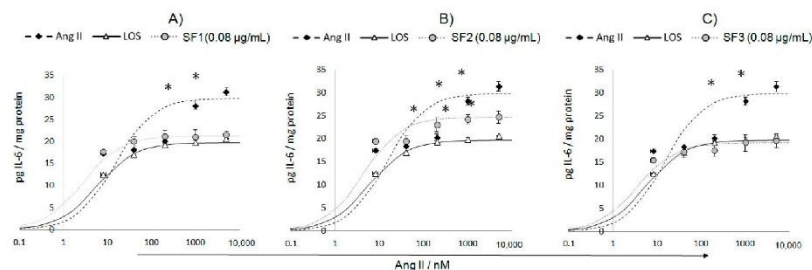


Figure 1. Cont.

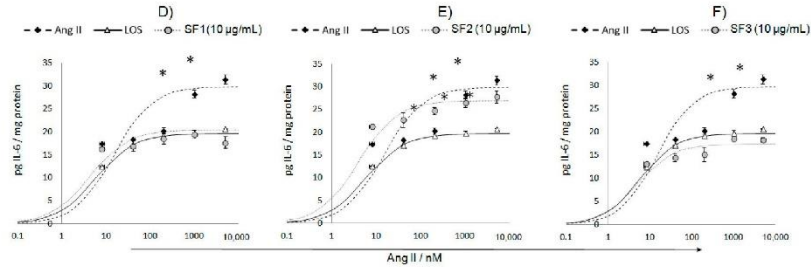


Figure 1. Concentration–response curves of the subfractions evaluated on Ang II (angiotensin II)-induced IL-6 (Interleukin 6) production, 12 h after treatment. (A–C) Effect of 0.08 µg/mL of SF1 (subfraction 1), SF2, and SF3; (D–F) Effect of 10 µg/mL of SF1, SF2, and SF3. Human microvascular endothelial cells-1 (HMEC-1) cells were co-cultured with the concentrations of Ang II specified above. Losartan (LOS) was used as a positive control. Data are reported as mean ± SD (standard deviation) and were analyzed by ANOVA and the post-hoc Tukey test. $n = 5$, * $p < 0.05$ vs. control (Ang II-free).

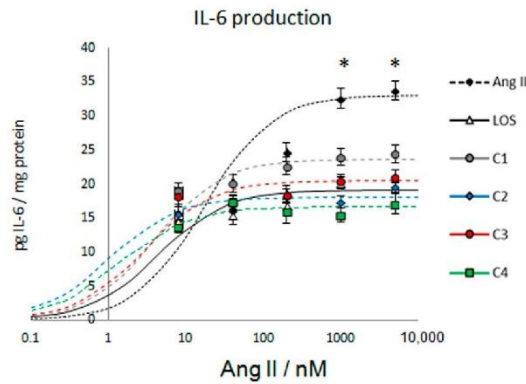


Figure 2. Concentration–response curves of the assessed combinations on Ang II-induced IL-6 production, 12 h after treatment. Losartan (LOS) was used as a positive control. C1 = 0.08 µg/mL of SF1 and 0.08 µg/mL of SF3; C2 = 0.08 µg/mL of SF1 and 10 µg/mL of SF3; C3 = 10 µg/mL of SF1 and 0.08 µg/mL of SF3; C4 = 10 µg/mL of SF1 and 10 µg/mL of SF3. Data are reported as mean ± SD and were analyzed by ANOVA and the post-hoc Tukey test. $n = 5$, * $p < 0.05$ vs. control (Ang II-free).

3.3. C4 Prevented E-Selectin and ICAM-1 Expression

Along with cytokine production, a well-known proinflammatory parameter induced by Ang II is the expression of adhesion molecules. Thus, the capacity of C4 to prevent the expression of E-selectin (Figure 3) and ICAM-1 (Supplementary Figure S1) was evaluated. Our results indicate that HMEC-1 cells expressed both adhesion molecules in the presence of Ang II 1000 and 5000 nM. This expression was diminished by C4, even at the highest Ang II concentration tested. Similar results were obtained with losartan.

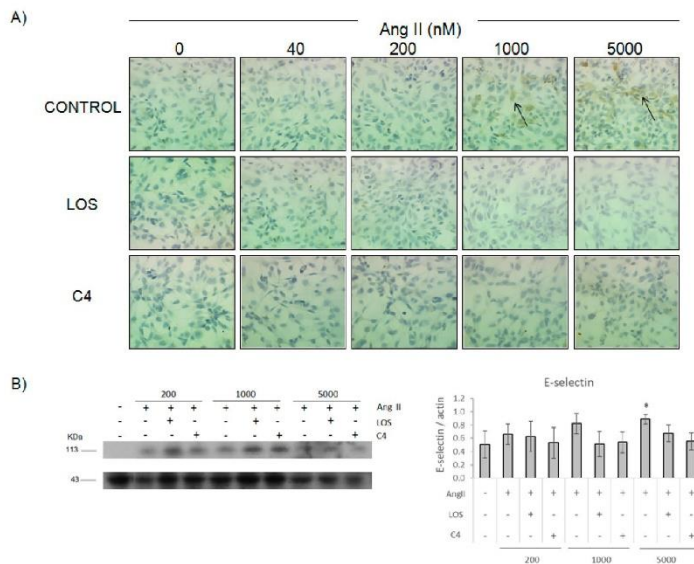


Figure 3. Effect of C4 on Ang II-induced E-selectin expression, 12 h after treatment. Immunocytochemistry (A). Western blot and expression relative to actin (B). Arrows indicate the E-selectin label. Microphotographs were taken with a 40× objective. LOS: Losartan; C4: Combination of SF1 and SF3, 10 µg/mL each. Data are reported as mean ± SD and were analyzed by ANOVA and the post-hoc Tukey test. *n* = 4, **p* < 0.05 vs. control (Ang II-free).

3.4. C4 Favored NO Bioavailability

The decrease in NO bioavailability is a key marker of oxidative stress induced by Ang II. Thus, we assessed the capacity of C4 to prevent a reduction in NO availability by the Griess reaction. As shown in Figure 4, Ang II caused a small but significant reduction in NO bioavailability; this effect was abolished by C4, maintaining this parameter at levels similar to Ang II-free and losartan controls.

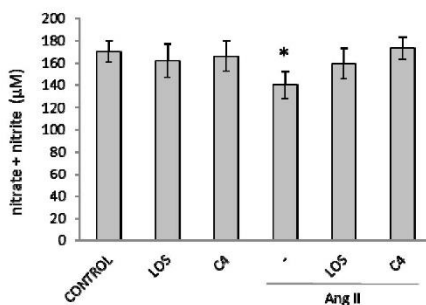


Figure 4. Effect of C4 on nitric oxide (NO) availability. HMEC-1 cells were stimulated with LOS or C4 or with Ang II 5000 nM plus LOS or C4. Data are reported as mean ± SD (standard deviation) and were analyzed by ANOVA and the post-hoc Tukey test. *n* = 5, **p* < 0.05 vs. control (Ang II-free). LOS: Losartan; C4: Combination of SF1 and SF3, 10 µg/mL each.

3.5. C4 Prevented Ang II-Induced ROS Increase

Ang II promotes oxidative stress by inducing the activation of enzymes such as NADH oxidase, the respiratory chain, eNOS, and xanthine oxidase. Thus, we evaluated the effect of C4 on the production of ROS by these enzymes. As shown in Figure 5, Ang II significantly increased the generation of ROS by the four enzymes assessed. C4 significantly prevented ROS formation, reducing the levels of NADH oxidase-derived ROS by 78.3%, a value similar to those obtained with losartan (85.5%) and the selective inhibitor DPI (66.1%; Figure 5, panels A,B). With respect to mitochondrial ROS, C4 decreased their levels by 83.4%, while the decrease due to losartan was 72.6%, and that due to antimycin was 87.1% (Figure 5, panels C,D). Additionally, C4 reduced ROS production by eNOS (74.5%; Figure 5, panels E,F) and xanthine oxidase (87.7%; Figure 5, panels G,H) to a similar extent as losartan (81.3% and 90.9%), L-NAME (60.7%), and allopurinol (89.5%).

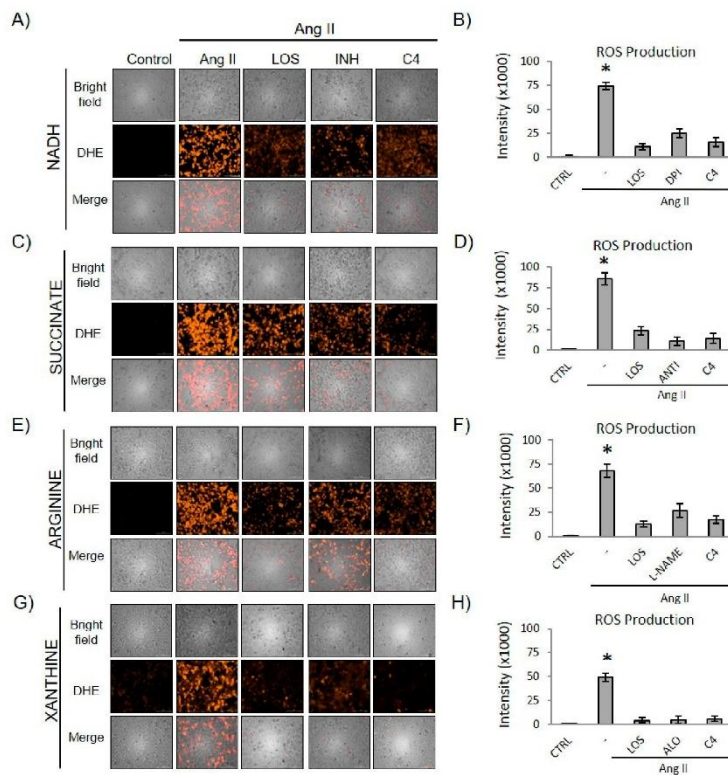


Figure 5. Effect of C4 on the production of $O_2\bullet^-$ from four different reactive oxygen species (ROS) sources, NADH (A,B), Succinate (C,D), Arginine (E,F) and Xanthine (G,H). HMEC-1 cells were stimulated with Ang II 5000 nM for 6 h. Representative microphotographs were taken with a 20 \times objective (A,C,E,G). Data are reported as mean \pm SD and were analyzed by ANOVA and the post-hoc Tukey test. $n = 5$, * $p < 0.05$ vs. control (Ang II-free), (B,D,G,H). LOS: Losartan; INH: Respective inhibitor; C4: Combination of SF1 and SF3, 10 μ g/mL each; DHE: Dihydroethidium; ROS: reactive oxygen species; ALO: allopurinol; CTRL: Control; DPI: Diphenyleneiodonium; NADH: nicotinamide adenine dinucleotide phosphate; ANTI: antimycin; L-NAME: N(ω)-nitro-L-arginine methyl ester.

3.6. Identification of Major Compounds

To identify the compounds in SF1 and SF3 with potential biological effects, a thin-layer chromatography assay was performed. The results (Supplementary Figure S2) indicated that amino acids are the major compounds in the subfractions. Mass spectrometry results are shown in Table 1 and Supplementary Figure S3. Positive ionization produced precursor peaks with molecular weights of 58.84, 133.93, 146.97, and 157.01 Da, matching the glycine, aspartic acid, lysine, and arginine standards, respectively, being the former the major amino acid in both subfractions. On the other hand, negative ionization produced a precursor peak with a molecular weight of 132.98 Da, corresponding to the asparagine standard.

Table 1. Amino acids identified in the subfractions SF1 and SF3 from *Cucumis sativus* aqueous fraction by mass spectrometry.

Ion Mode	Precursor Peak (m/z)	Amino Acid	Molecular Formula	Reported Bioactivity	References
Positive	58.84–58.88	Glycine	C ₂ H ₅ NO ₂	Reduces TNF- α , IL-6, COX-2, and NF- κ B. Improves the bioavailability of NO. Increases GSH, SOD, and eNOS.	[21–27]
Negative	132.98	Asparagine	C ₄ H ₈ N ₂ O ₃	Downregulates caspase-3, TNF- α , and TLR4 and its downstream signaling.	[28]
Positive	133.93–133.96	Aspartic acid	C ₄ H ₇ NO ₄	No activity reported.	-
Positive	146.97–147.00	Lysine	C ₆ H ₁₄ N ₂ O ₂	No activity reported.	-
Positive	175.01–175.06	Arginine	C ₆ H ₁₄ N ₄ O ₂	NO precursor. Reduces IL-17 and NF- κ B. Inhibits iNOS. Increases SOD and PI3K. Scavenger activity.	[29–32]

TNF- α : tumor necrosis factor α ; IL-6: Interleukin 6; COX-2: Cyclooxygenase-2; NF- κ B: Nuclear factor kappa B; NO: nitric oxide; GSH: Glutathione; SOD: superoxidase dismutase; eNOS: endothelial nitric oxide synthase; TLR4: Toll-like receptor 4; iNOS: Inducible nitric oxide synthase; PI3K: phosphoinositide 3-kinase.

4. Discussion

Inflammation and oxidative stress are strongly associated with the progression of endothelial dysfunction; in turn, the latter is the physiopathological substrate of several cardiovascular diseases with high impact on mortality indices such as myocardial infarction, cerebrovascular disease, and chronic renal failure [33–35]. Thus, treatments to control both conditions are emerging as key therapies against these high-impact diseases [1].

Whilst endothelial dysfunction was induced in HMEC-1 cells by adding Ang II to the culture medium in concentrations much larger than physiological levels, these concentrations proved to be the most effective conditions to induce endothelial dysfunction in vitro in this cell line. Several studies have used this Ang II dose-range to induce inflammatory and oxidative responses [36–40]. On the other hand, HMEC-1 cells were chosen as a model because they are employed in various studies to induce endothelial dysfunction, increasing the production of ROS and inflammatory factors [41–43].

The subfractions SF1, SF2, and SF3 from the *C. sativus* aqueous fraction were evaluated herein for their capacity to prevent Ang II-induced inflammation and oxidative stress in vitro. Losartan, an AT1R antagonist, is capable of inhibiting the downstream effects of its activation. Our findings indicate that SF1 and SF3 modulated several inflammatory and oxidative parameters as effectively as losartan. These results could be due to glycine and arginine, the main compounds detected in SF1 and SF3. Both amino acids have been reported to have anti-inflammatory effects since they inhibit the expression of IL-6, IL-1 β , IL-17, TNF- α , and cyclooxygenase 2, as well as macrophage infiltration; they also downregulate the activity of NF- κ B [21–24,29–31]. However, as shown by chromatography, other compounds, related to polysaccharides, were also present in the three subfractions; these compounds could also be exerting anti-inflammatory and anti-oxidant effects

as observed in other plants of the family Cucurbitaceae [44,45]. Thus, the overall effect of the assayed subfractions on endothelial dysfunction could be due to the combined effect of amino acids and polysaccharides present in these subfractions.

Ang II is an octapeptide; upon binding the receptor AT1R in the endothelial cell, it promotes the MAPK signaling cascade that leads to the activation of the inhibitor of NF- κ B (I κ B) kinase (IKK); in turn, IKK phosphorylates I κ B. After being phosphorylated, I κ B is degraded and NF- κ B translocates into the nucleus. NF- κ B is a transcription factor that controls the expression of genes involved in inflammation such as IL-6, E-selectin, and ICAM-1 [46]. It has been reported that when glycine binds its receptor (GlyR), which is an ionotropic or ligand-gated receptor, it contributes to hyperpolarizing cells such as macrophages, hindering its activation [25]. Other authors [21,47] have reported that this hyperpolarization inhibits the MAPK pathway. Then, the activation of NF- κ B induced by Ang II and its inflammatory consequences are blocked. GlyR is also expressed in endothelial cells [48,49]. Thus, glycine in SF1 and SF3 could interact with GlyR in endothelial cells, hyperpolarizing their membrane and blocking the Ang II-mediated MAPK signaling, and consequently inhibiting the activation of NF- κ B and the production of IL-6, E-selectin, and ICAM-1. L-Arginine is another amino acid which has exhibited anti-inflammatory properties in vivo and in vitro by diminishing the expression of inflammatory cytokines [29,50]. Recently, L-arginine was demonstrated to inhibit NF- κ B activation in Caco-2 cells by a mechanism involving its active transport by the cationic amino acid transporter CAT1. L-arginine also promotes the activity of eNOS to generate NO, increasing its bioavailability [50].

Ang II also has prooxidant effects and induces a decrease in NO bioavailability [5,13]. Upon binding AT1R, it triggers the signaling pathway that activates NADH oxidase to produce $O_2^{\bullet-}$ [4], increasing the intracellular concentration of this radical ion, and overcoming cellular antioxidant mechanisms (superoxide dismutase, catalase, and glutathione peroxidase). The increase of ROS also results in eNOS uncoupling, which switches its activity on the same substrate to produce further $O_2^{\bullet-}$ instead of NO, increasing the prooxidative environment [6]. The results herein reported demonstrated that a combination of SF1 and SF3 (C4), 10 μ g/mL each prevented the decrease in NO levels. Glycine and arginine, the main amino acid components in both subfractions, could be responsible for this effect along with the polysaccharide content (which could not be identified). Glycine has been reported to increase the expression of eNOS mRNA and of the protein itself; thus, more enzyme is available to produce NO [23,26]. On the other hand, the substrate of eNOS is arginine [29]; consequently, SF1 and SF3 could act by simultaneously increasing the levels of the enzyme and its substrate to synthesize NO. In addition, arginine stimulates the phosphoinositide 3-kinase/Akt pathway [30], which favors eNOS activation, the production of NO, and its vasodilatory effects, thus improving endothelial function. Besides, exogenously administered L-arginine has proved to be able to attenuate Ang II-mediated hypertension in rats, reversing the vasoconstrictor effects of Ang II and restoring renal excretory function, probably by mechanisms involving a cellular increase of L-arginine uptake and NO production [51]. Although both amino acids have been reported to exert anti-inflammatory and anti-oxidant effects, the particular combination of each one, along with the presence of other (not yet identified) trace components must be relevant to the overall effect herein reported, since not all combinations were equally efficient to control endothelial dysfunction. Indeed, the C4 combination was the most effective in controlling IL-6 production, a cytokine well known for its capacity to promote inflammation. Human supplementation with any of these two amino acids has been demonstrated to modulate vascular dysfunction [52,53], and these results may support the potential use of a standardized *C. sativus* fraction to control these pathological conditions.

As previously mentioned, Ang II promotes oxidative stress by increasing ROS production. There are many potential sources of ROS in cells, including NADH oxidases, mitochondria, xanthine oxidase, and the impaired eNOS [4]. We showed that treating HMEC-1 cells with C4 (a combination of SF1 and SF3) significantly decreased the Ang II-induced ROS production from these sources. These results could be due to the effect of the amino acid constituents of C4 to improve antioxidant

defenses. Arginine and glycine have been reported to increase the amount of copper/zinc superoxide dismutase (Cu/Zn-SOD), an antioxidant enzyme capable of reducing the superoxide ion to hydrogen peroxide [26,30]. On the other hand, arginine has a proven antioxidant activity by its capacity to scavenge free radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) [26]. In addition, glycine is a precursor of glutathione (glutamyl-cysteinyl-glycine), a tripeptide with antioxidative effects mediated by (i) a peroxidase-coupled reaction; (ii) regulation of the cellular sulfhydryl and redox balance; and (iii) regulation of the expression/activation of redox-sensitive transcription factors [24,32].

In conclusion, the particular combination of different concentrations of glycine, arginine, and polysaccharides present in the aqueous fractions of *C. sativus* could be potentially useful to treat various pathologies associated with inflammation and oxidative stress, such as endothelial dysfunction.

5. Conclusions

Overall, this study demonstrates that the combination C4 from the SF1 and SF3 subfractions of the *C. sativus* aqueous fraction was efficient in reducing Ang II-induced inflammatory factors and oxidative stress in HMEC-1 cells.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2072-6643/10/3/276/s1>, Supplementary Figure S1. Effect of C4 on Ang II-induced ICAM-1 expression, 12 h after treatment. Supplementary Figure S2. Thin-layer chromatography. Supplementary Figure S3. Mass spectrometry.

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Conflicts of Interest: The authors declare no conflict of interest.

References

- Endemann, D.H.; Schiffrin, E.L. Endothelial dysfunction. *J. Am. Soc. Nephrol.* **2004**, *15*, 1983–1992. [[CrossRef](#)] [[PubMed](#)]
- Jaimes, E.A.; Hua, P.; Tian, R.X.; Raj, L. Human glomerular endothelium: Interplay among glucose, free fatty acids, angiotensin II, and oxidative stress. *Am. J. Physiol. Renal Physiol.* **2010**, *298*, F125–F132. [[CrossRef](#)] [[PubMed](#)]
- Husain, K.; Hernandez, W.; Ansari, R.A.; Ferder, L. Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J. Biol. Chem.* **2015**, *6*, 209–217. [[CrossRef](#)] [[PubMed](#)]
- Wang, W.; Wang, S.; Yan, L.; Madara, P.; Del Pilar Cintron, A.; Wesley, R.A.; Danner, R.L. Superoxide production and reactive oxygen species signaling by endothelial nitric-oxide synthase. *J. Biol. Chem.* **2000**, *275*, 16899–16903. [[CrossRef](#)] [[PubMed](#)]
- González, J.; Valls, N.; Brito, R.; Rodrigo, R. Essential hypertension and oxidative stress: New insights. *World J. Cardiol.* **2014**, *6*, 353–366. [[CrossRef](#)] [[PubMed](#)]
- Li, H.; Förstermann, U. Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease. *Curr. Opin. Pharmacol.* **2013**, *13*, 161–167. [[CrossRef](#)] [[PubMed](#)]
- Gloire, G.; Legrand-Poels, S.; Piette, J. NF- κ B activation by reactive oxygen species: Fifteen years later. *Biochem. Pharmacol.* **2006**, *72*, 1493–1505. [[CrossRef](#)] [[PubMed](#)]
- Nakajima, S.; Kitamura, M. Bidirectional regulation of NF- κ B by reactive oxygen species: A role of unfolded protein response. *Free Radic. Biol. Med.* **2013**, *65*, 162–174. [[CrossRef](#)] [[PubMed](#)]
- Ma, X.; Dang, C.; Kang, H.; Dai, Z.; Lin, S.; Guan, H.; Liu, X.; Wang, X.; Hui, W. Saikosaponin-D reduces cisplatin-induced nephrotoxicity by repressing ROS-mediated activation of MAPK and NF- κ B signalling pathways. *Int. Immunopharmacol.* **2015**, *28*, 399–408. [[CrossRef](#)] [[PubMed](#)]

10. Ushio-Fukai, M.; Alexander, R.W.; Akers, M.; Yin, Q.; Fujio, Y.; Walsh, K.; Griendling, K.K. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J. Biol. Chem.* **1999**, *274*, 22699–22704. [[CrossRef](#)] [[PubMed](#)]
11. Costanzo, A.; Moretti, F.; Burgio, V.L.; Bravi, C.; Guido, F.; Levrero, M.; Puri, P.L. Endothelial activation by angiotensin II through NFκB and p38 pathways: Involvement of NFκB-inducible kinase (NIK), free oxygen radicals, and selective inhibition by aspirin. *J. Cell. Physiol.* **2003**, *195*, 402–410. [[CrossRef](#)] [[PubMed](#)]
12. Henke, N.; Schmidt-Ullrich, R.; Dechend, R.; Park, J.K.; Qadri, F.; Wellner, M.; Obst, M.; Gross, V.; Dietz, R.; Luft, F.C.; et al. Vascular endothelial cell-specific NF-κB suppression attenuates hypertension-induced renal damage. *Circ. Res.* **2007**, *101*, 268–276. [[CrossRef](#)] [[PubMed](#)]
13. Li, W.; Li, Z.; Chen, Y.; Li, S.; Lv, Y.; Zhou, W.; Liao, M.; Zhu, F.; Zhou, Z.; Cheng, X.; et al. Autoantibodies targeting AT1 receptor from patients with acute coronary syndrome upregulate proinflammatory cytokines expression in endothelial cells involving NF-κB pathway. *J. Immunol. Res.* **2014**, *2014*, 1–13. [[CrossRef](#)] [[PubMed](#)]
14. Bihl, J.C.; Zhang, C.; Zhao, Y.; Xiao, X.; Ma, X.; Chen, Y.; Chen, S.; Zhao, B.; Chen, Y. Angiotensin-(1–7) counteracts the effects of Ang II on vascular smooth muscle cells, vascular remodeling and hemorrhagic stroke: Role of the NFκB inflammatory pathway. *Vasc. Pharmacol.* **2015**, *73*, 115–123. [[CrossRef](#)] [[PubMed](#)]
15. De Sousa Falcão, H.; Leite, J.A.; Barbosa-Filho, J.M.; de Athayde-Filho, P.F.; de Oliveira Chaves, M.C.; Moura, M.D.; Ferreira, A.L.; de Almeida, A.B.; Souza-Brito, A.R.; de Fátima Formiga Melo Diniz, M.; et al. Gastric and duodenal antiulcer activity of alkaloids: A review. *Molecules* **2008**, *13*, 3198–3223. [[CrossRef](#)] [[PubMed](#)]
16. Kumar, D.; Kumar, S.; Singh, J.; Narender; Rashmi; Vashistha, B.; Singh, N. Free Radical Scavenging and Analgesic Activities of *Cucumis sativus* L. Fruit Extract. *J. Young Pharm.* **2010**, *2*, 365–368.
17. Mukherjee, P.K.; Nema, N.K.; Maity, N.; Sarkar, B.K. Phytochemical and therapeutic potential of cucumber. *Fitoterapia* **2013**, *84*, 227–236. [[CrossRef](#)] [[PubMed](#)]
18. Heidari, H.; Kamalinejad, M.; Noubarani, M.; Rahmati, M.; Jafarian, I.; Adiban, H.; Eskandari, M.R. Protective mechanisms of *Cucumis sativus* in diabetes-related models of oxidative stress and carbonyl stress. *Bioimpacts* **2016**, *6*, 33–39. [[CrossRef](#)] [[PubMed](#)]
19. Yamamoto, S.; Zhong, J.; Yancey, P.G.; Zuo, Y.; Linton, M.F.; Fazio, S.; Yang, H.; Narita, I.; Kon, V. Atherosclerosis following renal injury is ameliorated by pioglitazone and losartan via macrophage phenotype. *Atherosclerosis* **2015**, *242*, 56–64. [[CrossRef](#)] [[PubMed](#)]
20. Miranda, K.M.; Espey, M.G.; Wink, D.A. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric Oxide* **2001**, *5*, 62–71. [[CrossRef](#)] [[PubMed](#)]
21. Wang, H.D.; Lü, X.X.; Lu, D.X.; Qi, R.B.; Wang, Y.P.; Fu, Y.M.; Wang, L.W. Glycine inhibits the LPS-induced increase in cytosolic Ca²⁺ concentration and TNFα production in cardiomyocytes by activating a glycine receptor. *Acta Pharmacol. Sin.* **2009**, *30*, 1107–1114. [[CrossRef](#)] [[PubMed](#)]
22. Almanza-Perez, J.C.; Alarcon-Aguilar, F.J.; Blancas-Flores, G.; Campos-Sepulveda, A.E.; Roman-Ramos, R.; Garcia-Macedo, R.; Cruz, M. Glycine regulates inflammatory markers modifying the energetic balance through PPAR and UCP-2. *Biomed. Pharmacother.* **2010**, *64*, 534–540. [[CrossRef](#)] [[PubMed](#)]
23. Gómez-Zamudio, J.H.; García-Macedo, R.; Lázaro-Suárez, M.; Ibarra-Barajas, M.; Kumate, J.; Cruz, M. Vascular endothelial function is improved by oral glycine treatment in aged rats. *Can. J. Physiol. Pharmacol.* **2015**, *93*, 465–473. [[CrossRef](#)] [[PubMed](#)]
24. Amin, F.U.; Shah, S.A.; Kim, M.O. Glycine inhibits ethanol-induced oxidative stress, neuroinflammation and apoptotic neurodegeneration in postnatal rat brain. *Neurochem. Int.* **2016**, *96*, 1–12. [[CrossRef](#)] [[PubMed](#)]
25. Froh, M.; Thurman, R.G.; Wheeler, M.D. Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2002**, *283*, G856–G863. [[CrossRef](#)] [[PubMed](#)]
26. Ruiz-Ramírez, A.; Ortiz-Balderas, E.; Cardozo-Saldaña, G.; Diaz-Diaz, E.; El-Hafidi, M. Glycine restores glutathione and protects against oxidative stress in vascular tissue from sucrose-fed rats. *Clin. Sci.* **2014**, *126*, 19–29. [[CrossRef](#)] [[PubMed](#)]
27. Haddad, J.J.; Harb, H.L. L-γ-Glutamyl-L-cysteinyl-glycine (glutathione; GSH) and GSH-related enzymes in the regulation of pro- and anti-inflammatory cytokines: A signaling transcriptional scenario for redox(y) immunologic sensor(s)? *Mol. Immunol.* **2005**, *42*, 987–1014. [[CrossRef](#)] [[PubMed](#)]

28. Chen, S.; Liu, Y.; Wang, X.; Wang, H.; Li, S.; Shi, H.; Zhu, H.; Zhang, J.; Pi, D.; Hu, C.A.; et al. Asparagine improves intestinal integrity, inhibits TLR4 and NOD signaling, and differently regulates p38 and ERK1/2 signaling in weanling piglets after LPS challenge. *Innate Immun.* **2016**, *22*, 577–587. [[CrossRef](#)] [[PubMed](#)]
29. Hnia, K.; Gayraud, J.; Hugon, G.; Ramonatxo, M.; De La Porte, S.; Matecki, S.; Momet, D. L-arginine decreases inflammation and modulates the nuclear factor- κ B/matrix metalloproteinase cascade in mdx muscle fibers. *Am. J. Pathol.* **2008**, *172*, 1509–1519. [[CrossRef](#)] [[PubMed](#)]
30. Ren, W.; Yin, J.; Wu, M.; Liu, G.; Yang, G.; Xion, Y.; Su, D.; Wu, L.; Li, T.; Chen, S.; et al. Serum amino acids profile and the beneficial effects of L-arginine or L-glutamine supplementation in dextran sulfate sodium colitis. *PLoS ONE* **2014**, *9*, 1–13. [[CrossRef](#)] [[PubMed](#)]
31. Morais, S.R.; Brito, V.G.B.; Mello, W.G.; Oliveira, S.H.P. L-arginine modulates inflammation and muscle regulatory genes after a single session of resistance exercise in rats. *Scand. J. Med. Sci. Sports* **2018**, *28*, 425–435. [[CrossRef](#)] [[PubMed](#)]
32. Ahmad, A.; Sattar, M.Z.; Rathore, H.A.; Hussain, A.I.; Khan, S.A.; Fatima, T.; Afzal, S.; Abdullah, N.A.; Johns, E.J. Antioxidant activity and free radical scavenging capacity of L-arginine and NaHS: A comparative in vitro study. *Acta Pol. Pharm.* **2015**, *72*, 245–252. [[PubMed](#)]
33. Tanaka, S.; Masuda, T.; Kamiya, K.; Hamazaki, N.; Akiyama, A.; Kamada, Y.; Maekawa, E.; Noda, C.; Yamaoka-Tojo, M.; Ako, J. A single session of neuromuscular electrical stimulation enhances vascular endothelial function and peripheral blood circulation in patients with acute myocardial infarction. *Int. Heart J.* **2016**, *57*, 676–681. [[CrossRef](#)] [[PubMed](#)]
34. Kolmos, M.; Krawczyk, R.S.; Kruuse, C. Effect of high-intensity training on endothelial function in patients with cardiovascular and cerebrovascular disease: A systematic review. *SAGE Open Med.* **2016**, *4*, 1–13. [[CrossRef](#)] [[PubMed](#)]
35. Mitsides, N.; Cornelis, T.; Broers, N.J.H.; Diederens, N.M.P.; Brechley, P.; van der Sande, F.M.; Schalkwijk, C.G.; Kooman, J.P.; Mitra, S. Extracellular overhydration linked with endothelial dysfunction in the context of inflammation in haemodialysis dependent chronic kidney disease. *PLoS ONE* **2017**, *12*, 1–15. [[CrossRef](#)] [[PubMed](#)]
36. Pastore, L.; Tessitore, A.; Martinotti, S.; Toniato, E.; Alesse, E.; Bravi, M.C.; Ferri, C.; Desideri, G.; Gulino, A.; Santucci, A. Angiotensin II stimulates intercellular adhesion molecule-1 (ICAM-1) expression by human vascular endothelial cells and increases soluble ICAM-1 release in vivo. *Circulation* **1999**, *100*, 1646–1652. [[CrossRef](#)] [[PubMed](#)]
37. Gräfe, M.; Auch-Schwelk, W.; Zakrzewicz, A.; Regitz-Zagrosek, V.; Bartsch, P.; Graf, K.; Loebe, M.; Gaehgtgens, P.; Fleck, E. Angiotensin II-induced leukocyte adhesion on human coronary endothelial cells is mediated by E-selectin. *Circ. Res.* **1997**, *81*, 804–811. [[CrossRef](#)] [[PubMed](#)]
38. Zuo, Z.; Zuo, P.F.; Tian, X.Y.; Ma, G.S. Adiponectin ameliorates angiotensin II-induced vascular endothelial damage. *Cell Stress Chaperones* **2014**, *19*, 705–713. [[CrossRef](#)]
39. Chen, Y.; Lin, J.R.; Gao, P.J. Mitochondrial division inhibitor Mdivi-1 ameliorates angiotensin II-induced endothelial dysfunction. *Sheng Li Xue Bao* **2016**, *68*, 669–676. [[PubMed](#)]
40. Cao, Y.; Zheng, L.; Liu, S.; Peng, Z.; Zhang, S. Total flavonoids from *Plumula Nelumbinis* suppress angiotensin II-induced fractalkine production by inhibiting the ROS/NF- κ B pathway in human umbilical vein endothelial cells. *Exp. Ther. Med.* **2014**, *7*, 1187–1192. [[CrossRef](#)] [[PubMed](#)]
41. Zhu, X.; Lei, Y.; Tan, F.; Gong, L.; Gong, H.; Yang, W.; Chen, T.; Zhang, Z.; Cai, W.; Hou, B.; et al. Vaccarin protects human microvascular endothelial cells from apoptosis via attenuation of HDAC1 and oxidative stress. *Eur. J. Pharmacol.* **2018**, *818*, 371–380. [[CrossRef](#)] [[PubMed](#)]
42. Kurpios-Piec, D.; Grosicka-Maciąg, E.; Woźniak, K.; Kowalewski, C.; Kiernozek, E.; Szumiło, M.; Rahden-Staroń, I. Thiram activates NF- κ B and enhances ICAM-1 expression in human microvascular endothelial HMEC-1 cells. *Pestic. Biochem. Physiol.* **2015**, *118*, 82–89. [[CrossRef](#)] [[PubMed](#)]
43. Mudaliar, H.; Pollock, C.; Ma, J.; Wu, H.; Chadban, S.; Panchapakesan, U. The role of TLR2 and 4-mediated inflammatory pathways in endothelial cells exposed to high glucose. *PLoS ONE* **2014**, *9*, e108844. [[CrossRef](#)] [[PubMed](#)]
44. Song, Y.; Ni, Y.; Hu, X.; Li, Q. Effect of phosphorylation on antioxidant activities of pumpkin (*Cucurbita pepo*, *Lady godiva*) polysaccharide. *Int. J. Biol. Macromol.* **2015**, *81*, 41–48. [[CrossRef](#)] [[PubMed](#)]



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Acetone fraction from *Sechium edule* (Jacq.) S.w. edible roots exhibits anti-endothelial dysfunction activity



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ABSTRACT

Ethnopharmacological relevance: A recent ethnomedical survey on medicinal plants grown in Mexico revealed that *Sechium edule* (Jacq.) Sw. (Cucurbitaceae) is one of the most valued plant species to treat cardiovascular diseases, including hypertension. Fruits, young leaves, buds, stems, and tuberous roots of the plant are edible. Considering that endothelial dysfunction induced by Angiotensin II plays an important role in the pathogenesis of hypertension and is accompanied by a prooxidative condition, which in turn induces an inflammatory state, vascular remodeling, and tissue damage, and that *S. edule* has been reported to possess antioxidant, anti-inflammatory and antihypertensive activity, its capability to control endothelial dysfunction was also assessed.

Aim of the study: To assess *in vivo* the anti-endothelial dysfunction activity of the acetone fraction (rSe-ACE) of the hydroalcoholic extract from *S. edule* roots.

Materials and methods: Endothelial dysfunction was induced in female C57BL/6J mice by a daily intraperitoneal injection of angiotensin II for 10 weeks. Either rSe-ACE or losartan (as a control) were co-administered with angiotensin II for the same period. Blood pressure was measured at weeks 0, 5, and 10. Kidney extracts were prepared to determine IL1 β , IL4, IL6, IL10, IL17, IFN γ , TNF α , and TGF β levels by ELISA, along with the prooxidative status as assessed by the activity of antioxidant enzymes. The expression of ICAM-1 was evaluated by immunohistochemistry in kidney histological sections. Kidney and hepatic damage, as well as vascular tissue remodeling, were studied.

Results: The rSe-ACE fraction administered at a dose of 10 mg/kg was able to control hypertension, as well as the prooxidative and proinflammatory status in kidney as efficiently as losartan, returning mice to normotensive levels. Additionally, the fraction was more efficient than losartan to prevent liver and kidney damage. Phytochemical characterization identified cinnamic acid as a major compound, and linoleic, palmitic, and myristic acids as the most abundant non-polar components in the mixture, previously reported to aid in the control of hypertension, inflammation, and oxidative stress, three important components of endothelial

Abbreviations: Ab, antibody; AGII, angiotensin II; AT1R, angiotensin II receptor type 1; AT2R, angiotensin II receptor type 2; BHA, branch of hepatic artery; CMC, carboxymethylcellulose; DAP, diastolic arterial pressure; DHE, dihydroethidium oxidation; ED, endothelial dysfunction; Eth, ethidium; eNO, endothelium-derived nitric oxide; eNOS, endothelial nitric oxide synthase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; ICAM, intercellular adhesion molecule; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; LDL, low-density lipoprotein; L-NAME, NG-nitro-L-arginine methyl ester; MDA, malondialdehyde; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; O $_2^{\cdot-}$, superoxide anion; ONOO $^{\cdot-}$, peroxynitrite; PAS, periodic acid-Schiff; PDGF, platelet-derived growth factor; RI, renal insufficiency; RT, room temperature; ROS, reactive oxygen species; rSe-ACE, acetone *Sechium edule* fraction; rSe-HA, hydroalcoholic root extract; SAH, systemic arterial hypertension; SAP, systolic arterial pressure; SOD, superoxide dismutase; TNF, tumor necrosis factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; VLDL, very-low-density lipoprotein; VSMC, vascular smooth muscle cells; XO, xanthine oxidase

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dysfunction.

In conclusion: this study demonstrated that rSe-ACE has anti-endothelial dysfunction activity in an experimental model and highlights the role of cinnamic acid and fatty acids in the observed effects.

1. Introduction

Endothelial dysfunction (ED) is the result of a misbalance in the synthesis, release, and/or effect of several factors in endothelial cells. It is indicative of vascular damage and it plays a critical role in the pathogenesis of cardiovascular disorders like hypertension, renal dysfunction, and cerebrovascular diseases, which are leading causes of death worldwide (Schmitt and Dirsch, 2009). ED has a multifactorial origin that may be associated to alterations in the levels of glucose, triglycerides, LDL, VLDL, leptin, ROS, endothelin-1, vascular endothelial growth factor, C-reactive protein, and IL-6 in blood. ED is characterized by a vasoconstrictive, prooxidative, proinflammatory, prothrombotic, and proliferative status and by vascular remodeling (Endemann and Schiffrin, 2004), being the liver and kidney the organs most frequently affected (Bosch et al., 2010; Henke et al., 2007). AGII, part of the renin-angiotensin-aldosterone system, is critical in the development of this pathology (Adya et al., 2015; Husain et al., 2015; Jaimes et al., 2010; Liu et al., 2015). AGII binds to its receptors (AT1R or AT2R) in endothelial cells and induces the coupling of NAD(P)H oxidase, leading to the production of the superoxide anion (O_2^-). In turn, superoxide can act at different levels promoting an inflammatory and prooxidative status. Superoxide can bind to nitric oxide (NO) to form peroxynitrite (ONOO⁻), which causes inflammation secondary to a non-enzymatic conversion of arachidonic acid into prostaglandins. It can also uncouple endothelial nitric oxide synthase (eNOS), so the enzyme will stop synthesizing NO, which is important for relaxation of the vessels, and will synthesize O_2^- instead. The latter can act as a second messenger, inducing the expression of NF- κ B, leading to the production of proinflammatory cytokines, chemokines, and adhesion molecules (Jaimes et al., 2010; Marchesi et al., 2008; Nishida et al., 2010; Schramm et al., 2012). Additionally, AGII induces the production of several growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF), all of which induce the proliferation of vascular smooth muscle cells (VSMC) and extracellular matrix accumulation, leading to vascular remodeling (Gorman et al., 2014; Intengan and Schiffrin, 2001), which further contributes to hypertension.

Sechium edule, a member of the Cucurbitaceae family, is a Mexican plant cultivated since pre-Columbian times. Ethnomedical data indicate that it has been used to treat atherosclerosis, and as a hypotensive (Argueta et al., 1994; Ibarra-Alvarado et al., 2010). In Mexico, it is one of the most valued plants used to treat cardiovascular ailments, including hypertension (Ibarra-Alvarado et al., 2010). In previous studies, a hydroalcoholic extract from *S. edule* roots and its organic fractions showed antihypertensive activity; in particular, the acetone fraction had a relaxant effect on blood vessels (Lombardo-Earl et al., 2014; Lozoya, 1980). Herein, the study of this fraction was extended to its capability of controlling ED and hypertension in chronic conditions induced by AGII. Additionally, its antioxidant and antiinflammatory properties were determined, as well as its capacity to prevent tissue damage.

2. Materials and methods

2.1. Plant material and fraction preparation

Sechium edule (Jacq.) Sw. roots were collected in the community of Tuxpanguillo, Veracruz, Mexico (18°47'00.5" N, 97°00'17.5" W, at 1721 m above mean sea level) in a period from April through May. Plant material was identified by Abigail Aguilar-Contreras (IMSSM,

National Medical Center, Mexico City). Voucher specimens were stored at this site for future reference (IMSSM-15549). The name of the plant (*Sechium edule*) was confirmed to be included in www.theplantlist.org (The plant list, 2013). Roots (2.3 kg) were dehydrated by freeze-drying and ground in a Pulvex electric mill until particles less than 4 mm in diameter were obtained. To obtain the hydroalcoholic root extract (rSe-HA), an exhaustive maceration process was conducted with an ethanol/water mixture (60:40) in a final volume of 11.5 L at room temperature (RT). This extract was concentrated by distillation under reduced pressure and controlled temperature, and then dried by freeze-drying. rSe-HA (92 g) was suspended in acetone (16 g) for 24 h (two times); upon filtration, the liquid phase was concentrated in a Heidolph rotatory evaporator (Laborota 4000), yielding the acetone fraction (rSe-Ace).

2.2. GC-MS analysis

The chemical composition of the acetone fraction was determined by gas chromatography. The chromatograph was coupled with a quadrupole mass detector set in electron impact mode at 70 eV. Volatile compounds were resolved in a HP 5MS capillary column (25 m long, 0.2 mm i.d., 0.3 μ m film thickness). The oven temperature was set at 40 °C for 2 min, then programmed to rise from 40 °C to 260 °C at a speed of 10 °C/min, and then kept at 260 °C for 20 min. Mass detector parameters were set as follows: interphase temperature 200 °C and mass acquisition range 20–550. Injector and detector temperatures were set to 250 °C and 280 °C, respectively. One microliter of each fraction (3 mg/ml) was injected in splitless mode. The flow rate for the carrier gas (helium) was set to 1 ml/min. Volatile compounds were identified by comparing their mass spectra with those in the NIST 1.7 library.

2.3. HPLC analysis

HPLC data were acquired in a Waters 2695 separation module with a photodiode array detector and analyzed with the Empower Pro software (Waters Corporation, USA). Chemical separation was achieved using a Supelcosil LC-F column (4.6 mm \times 250 mm i.d., 5- μ m particle size) (Sigma-Aldrich, Bellefonte, USA). The mobile phase consisted of a trifluoroacetic acid aqueous solution 0.5% (solvent A) and acetonitrile (solvent B). A solvent gradient was set as follows: 0–1 min, 0% B; 2–3 min, 5% B; 4–20 min, 30% B; 21–23 min, 50% B; 14–15 min; 24–25 min, 80% B; 26–27 min, 100% B; 28–30 min, 0% B. Flow rate was maintained at 0.9 ml min⁻¹, and sample injection volume was 10 μ l. Absorbance was measured at 330 nm. Cinnamic acid was identified by comparing retention times and UV spectra with those of reference standards (Sigma-Aldrich, St Louis Mo, USA).

2.4. Animals and experimental groups

Female C57BL/6J mice (10-week-old) obtained from our animal facilities were used. Four groups of five mice each were housed and maintained under pathogen-free conditions in the animal facility at the Faculty of Medicine of the Autonomous University of Morelos (FM-UAEM) under constant temperature (21–23 °C) and humidity (45–50%) conditions, with a 12-h light/dark cycle. All experiments followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experimental protocols were reviewed and approved by the Ethical Committee for the Care and Use of Laboratory Animals (Permit No. 005/2011) at the FM-UAEM.

Control mice were treated with saline solution only. The three

remaining groups were treated with AGII (i.p., 0.1 µg/kg daily for 10 weeks), either alone or coadministered with losartan (10 mg/kg) diluted in carboxymethylcellulose (CMC) 0.2% (Bastaki et al., 2017) or rSe-ACE (10 mg/kg) diluted in water orally for 10 weeks. Mice were regarded as hypertensive when blood pressure increased by 15% or more with respect to baseline blood pressure (systolic or diastolic). Mice were killed by CO₂ inhalation and exsanguinated at the end of the experiment.

2.5. Tissue preparation for biochemical analysis

Kidneys were homogenized in ice-cold HEPES buffer (HEPES 25 mM, EDTA 1 mM, and phenylmethylsulfonyl fluoride [PMSF] 0.1 mM). Homogenates were centrifuged at 6000 × g for 5 min at 4 °C and supernatants were recovered. Protein concentration was determined by the Lowry method.

2.5.1. Measurement of O₂⁻ production

Dihydroethidium oxidation (DHE) to ethidium (Eth) was used to measure O₂⁻ production (Satoh et al., 2005). Briefly, supernatants (10 µl) were incubated with DHE (0.02 mM), salmon testis DNA (0.5 mg/ml), and the corresponding substrate for xanthine oxidase (XO), mitochondrial respiratory enzymes, or nitric oxide synthase (NOS). Eth-DNA fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 610 nm at 37 °C for 30 min using a multimode microplate reader (Synergy HT, Biotek, Winooski, VT). Xanthine (0.1 mM) was used as a substrate for XO, and allopurinol (0.2 mM) was used as an inhibitor. Succinate (5 mM) was used as a substrate for mitochondrial O₂⁻ production, and antimycin (0.05 mM) was used to block the respiratory chain. L-arginine (L-Arg, 1 mM) was used as a substrate for NOS, while NG-nitro-L-arginine methyl ester (L-NAME, 1 mM) was used to block NOS activity. A blank without sample was run to subtract background fluorescence from each sample reading. Enzyme activity is expressed with respect to control readings.

2.5.2. Activity of antioxidant enzymes

2.5.2.1. Glutathione peroxidase assay. The activity of renal glutathione peroxidase (GPx) was assayed by the method of Lawrence and Burk (1976) with some modifications (Satoh et al., 2005), measuring NADPH disappearance by optical density (OD) reading at 340 nm. GPx uses GSH to reduce tert-butyl hydroperoxide, thereby producing GSSG, which is readily reduced to GSH by GR using NADPH as a reducing equivalent donor. Blank reactions with the sample replaced by distilled water were subtracted from each assay reading. Data were expressed as units per mg protein.

2.5.2.2. Glutathione reductase assay. The activity of glutathione reductase (GR) was assayed using oxidized glutathione as a substrate (Carlberg and Mannervik, 1975). Briefly, GR reduces GSSG to GSH at the expense of NADPH; the disappearance of NADPH can be detected by reading OD at 340 nm.

2.5.2.3. Glutathione-S-transferase assay. The activity of glutathione-S-transferase (GST) was evaluated following the method of Habig et al. (1974). The reaction mixture consisted of potassium phosphate buffer 50 mM (pH 6.5), reduced glutathione 2 mM, and 1-chloro-2,4-dinitrobenzene (CDNB) 1 mM. Supernatants (0.02 ml) were added to 0.98 ml of the reaction mixture, and absorbance was recorded at 340 nm for 3 min. Enzymatic activity was calculated as µmol CDNB conjugate formed/min/mg protein using a molar absorptivity of 9.6×10^3 M/cm.

2.5.2.4. Superoxide dismutase assay. The total activity of Superoxide Dismutase (SOD) was evaluated using a previously reported method (Oberley and Spitz, 1984). Briefly, a competitive inhibition assay was

performed using a xanthine-XO system to reduce nitroblue tetrazolium (NBT). The percentage of NBT reduction in the sample-free control tube was 100%. The amount of protein that inhibited NBT reduction by 50% of the maximum reading was defined as one unit of SOD activity. Results were expressed as U/mg protein.

2.5.2.5. Lipid peroxidation assay. The content of malondialdehyde (MDA) was measured using a standard curve of tetramethoxypropane. Tissue supernatants (0.2 ml) were added to 0.65 ml of 1-methyl-2-phenylindole 10 mM in an acetonitrile/methanol mixture (3:1). The reaction was started by adding 0.15 ml of HCl 37% and incubated for 40 min at 45 °C. Then, the samples were centrifuged at 3000 × g for 5 min. OD was measured at 586 nm (Gérard-Monnier et al., 1998). Data were expressed as nmol MDA/mg protein.

2.6. Blood pressure measurement

Mice were anesthetized with xylazine (10 mg/kg i.p.); then, systolic and diastolic arterial pressure data were recorded by the tail-cuff technique, using a LE5002 Panlab Non-Invasive Blood Pressure System (Letica). Blood pressure was recorded at the beginning of the experiment (baseline) and every five weeks until the end of the experiment. To avoid circadian variations (Marques et al., 2011), all measurements were performed between 11:00 a.m. and 15:00 p.m.

2.7. Cytokine detection by ELISA

Kidneys were weighed and frozen at -80 °C until used. The organs were macerated in a frozen mortar with ice-cold PBS-PMSF (0.1%) 1:5 w/v. The suspensions were centrifuged, and the supernatants were recovered and frozen at -20 °C until used. Various ELISA kits were used to determine cytokine concentration, following manufacturer's instructions. Mouse IL1β, IL4, IL6, IL10, IFNγ, and TNFα ELISA kits were purchased from OptEIA™ BD, and Mouse IL17A and TGFβ ELISA kits were purchased from Applied Biosystems. Briefly, 96-well flat-bottomed ELISA plates were coated with the respective capture antibody and incubated overnight at 4 °C in carbonate buffer (pH 9.6). Non-specific binding sites were blocked by incubating for 30 min at RT with PBS-5% fetal bovine serum. Aqueous kidney extracts were added and incubated for 2 h at RT. Then, the plates were incubated with the corresponding detection anti-cytokine-HRP antibodies for 30 min at RT. Bound complexes were detected by reaction with tetramethylbenzidine substrate after 30 min of incubation in the darkness. The reaction was stopped with H₂SO₄ 2 N and absorbance was measured at 450 nm at 37 °C in an ELISA plate reader (VERSAmax Molecular Devices). Cytokine concentration was calculated according to standard curves for each cytokine and reported as pg/mg protein.

2.8. Histopathology

After the last blood pressure measurement, mice were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and perfused with ice-cold PBS (NaCl 140 mM, KCl 2 mM, and K₂HPO₄ 1.15 mM). Kidneys and livers were removed. The organs were fixed in Zamboni solution (formaldehyde 2.0%, picric acid 0.2%, pH 7.0). Tissues were then dehydrated and embedded in paraffin. Tissue sections (5 µm) were transferred to poly-L-lysine-coated slides (Sigma) before being deparaffinized and rehydrated. For histopathological studies, the slides were stained with Masson trichrome stain (kidney and liver) or periodic acid-Schiff (PAS) stain (kidney). Masson trichrome method combines hematoxylin stain with a cytoplasmic stain, and a selective stain for connective tissue (Goldner, 1938). PAS stain detects polysaccharides (glycogen and mucosal substances such as glycoproteins, glycolipids and mucins) in tissues to identify alterations in the basement membrane (Feldman and Christian, 1981), which was used to determine the expansion of the mesangial matrix by the presence of increased amounts of periodic

acid-Schiff positive material in the mesangial region (Raj et al., 1984). All slides were observed under a Nikon ECLIPSE 80i microscope and analyzed using the Metamorph software, v. 6.1.

Glomerulosclerosis or hyalinization was defined as the disappearance of cellular elements from the tuft, collapse of capillary lumens, and folding of the glomerular basement membrane with entrapment of amorphous material, as proposed by Raj et al. (1984). Glomerular injury was also analyzed according to the method reported by the same author, with minor modifications. Briefly, 50–60 cortical glomeruli were evaluated in PAS-stained kidney slides from each group under a 100 × objective. Digitalized images were analyzed with the Metamorph software, v. 6.1. Mesangial area was determined by subtracting the capillary area from the total area. These values were expressed as percentage with respect to the total area.

Vascular remodeling was evaluated in the portal triad in Masson-stained liver slides. The total area and the luminal area of the branch of hepatic artery (BHA) were measured in 10 slides per group under the 40 × objective. The area corresponding to vessel thickness was determined by subtracting these two values. Lumen percentage, media thickness percentage, and media/lumen ratio were calculated to assess vascular remodeling (Intengan and Schiffrin, 2001).

2.9. Immunohistochemistry

Tissue slides were deparaffined, rehydrated, and incubated with H₂O₂ 3%, albumin 5%, and Tween 20-PBS 1%. After treatment, tissue sections were incubated overnight with rat anti-mouse ICAM-1

(eBioscience) diluted 1:100 in albumin 0.1% and Tween 20-PBS 0.05%. After wash with PBS, tissues were incubated with 50 µl of biotinylated goat anti-rat IgG (MP Biomedicals) antibody, followed by HRP-labeled streptavidin solution (MP Biomedicals) at 37 °C for 30 min, and developed with 3,3 diaminobenzidine (ZYMED, San Francisco, CA). The slides were counterstained with hematoxylin and photographed using a Nikon ECLIPSE 80i microscope. The images were analyzed with the Metamorph software, v. 6.1.

2.10. Statistics

Data are expressed as mean ± SD. Groups or treatments were compared by one-way ANOVA with a post-hoc Tukey-Kramer test. Differences were considered significant when *P* < 0.05.

3. Results

3.1. Identification of the major polyphenolic compounds

The HPLC fingerprint of the bioactive fraction (Fig. 1A–B) shows cinnamic acid (12.4 min; λ = 205, 280 nm) as its major chemical constituent. The chemical structure of this aromatic acid was corroborated by comparison of nuclear magnetic data of the compound isolated from *S. edule* with previously described constituents (Lombardo-Earl et al., 2014).

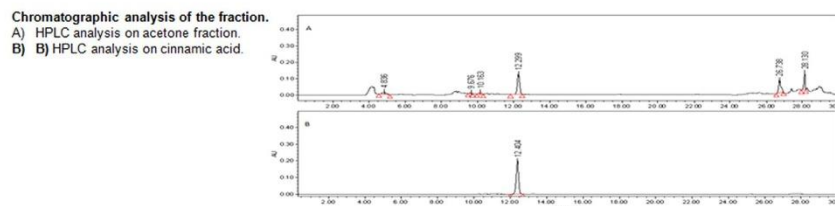


Table 1. Non-Polar phytochemicals identified in two retention times (RT) of the acetonitrile root extract of *Sechium edule* by GC-MS analysis

Retention Time	Peak No.	Corresponding compound	MW	RT	Molecular formula	Peak Area (%)	Reported Bioactivity
26.7 RT	1	Tetradecanoic acid (Myristic acid)	228.37	29.9	C ₁₄ H ₂₈ O ₂	4.3	Antioxidant, Hypocholesterolemic, anti-tumoral growth, renal protective effect
	2	Pentadecanoic acid	242.4	31.5	C ₁₅ H ₃₀ O ₂	3.99	Activation of PPARγ and PPARβ, regulation of energy catabolism, lipid homeostasis and fatty acid oxidation (FAO).
	3	11-Z-Hexadecenoic acid	254.4	33.6	C ₁₆ H ₃₀ O ₂	7.8	Antimicrobial and antioxidant activity.
	4	Hexadecanoic acid (palmitic acid)	256.4	34.08	C ₁₆ H ₃₂ O ₂	22.4	Antioxidant, Hypocholesterolemic.
	5	9,12-Octadecadienoic acid (Linoleic acid)	280.4	37.3	C ₁₈ H ₃₄ O ₂	30.4	Anti-inflammatory, Cox 1 and Cox2 inhibition, Hypocholesterolemic.
	6	9,12-Octadecadienyl chloride (Linoleic acid chloride)	298.89	37.4	C ₁₈ H ₃₃ ClO	11.1	No activity reported.
	7	Hexadecanoic acid, 2-hydroxy-1-(ethyl ester (2-Palmitoylglycerol)	330.6	43.35	C ₁₈ H ₃₆ O ₄	19.9	No activity reported.
28.1 RT	1	(M)-9-Oxononanoic acid	172.2	23.9	C ₉ H ₁₆ O ₃	10.4	No activity reported.
	2	Tetradecanoic acid (Myristic acid)	228.37	30.1	C ₁₄ H ₂₈ O ₂	28.8	Antioxidant, Hypocholesterolemic.
	3	14-Pentadecenoic acid	240.38	31.55	C ₁₅ H ₃₀ O ₂	5.6	Antioxidant
	4	Pentadecanoic acid	242.4	31.6	C ₁₅ H ₃₀ O ₂	2.9	Activation of PPARγ and PPARβ, regulation of energy catabolism, lipid homeostasis and FAO
	5	11-Z-Hexadecenoic acid	254.4	33.7	C ₁₆ H ₃₀ O ₂	22.9	Antimicrobial and antioxidant activity.
	6	Hexadecanoic acid (palmitic acid)	256.4	34.0	C ₁₆ H ₃₂ O ₂	7.5	Antioxidant, Hypocholesterolemic.
	7	9,12-Octadecadienoic acid (Linoleic acid)	280.4	37.2	C ₁₈ H ₃₄ O ₂	10.4	Anti-inflammatory, Cox 1 and Cox2 inhibition, Hypocholesterolemic.
	8	9,12,15, Octadecatrienoic acid, methyl ester (Linolenic acid methyl ester)	282.4	37.3	C ₁₈ H ₃₄ O ₂	12.0	Anti-inflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective, Antihistaminic, Anticanceric, Antiarthritic, Anticoronary.
	9	Hexadecanoic acid, 2-hydroxy-1-(ethyl ester (2-Palmitoylglycerol)	330.6	43.35	C ₁₈ H ₃₆ O ₄	1.45	No activity reported.

Fig. 1. Chromatographic analysis of the fraction. A) HPLC analysis of the acetone fraction. B) HPLC analysis of cinnamic acid. The HPLC fingerprint of the bioactive fraction identifies cinnamic acid (12.4 min; λ = 205, 280 nm) as its major chemical constituent. Table 1 shows other non-polar phytochemicals identified in two retention times (RT) of the acetonitrile root extract of *Sechium edule* by GC-MS analysis.

3.2. Identification of non-polar compounds

Chemical analysis of the less polar composition by GC-MS identified palmitic acid, linoleic acid, linoleic acid chloride, 11-Z-hexadecenoic acid, 2-palmitoylglycerol, and linolenic acid methyl ester, among other fatty acids (Table 1).

3.3. rSe-ACE prevents the production of O_2^-

The O_2^- ion can be produced in the cell through different metabolic pathways, which imply different enzymes and substrates. To assess whether rSe-ACE was able to prevent O_2^- production and determine the main enzymes involved in the production of this radical ion, the capacity of kidney extracts to oxidize dihydroethidium (DHE) under different conditions was evaluated. Protein extracts were obtained from all mice groups and were exposed to a set of substrates and enzyme inhibitors before measuring O_2^- production (Fig. S.1). Only when kidney extracts were exposed to succinate, the percent change in production O_2^- increased significantly, 233.2 ± 26.7 in the AGII-treated group and 100 ± 1.7 in the control group ($P < 0.01$), a nearly 2.5-fold increase. Extracts from AGII/rSe-ACE- or AGII/losartan-treated mice did not show this increase (120.9 ± 36.7 and 167.7 ± 49.7 , respectively), being similar to the normotensive control group. On the other hand, when homogenates were incubated with succinate and antimycin, a mitochondrial respiration inhibitor, a 0.67-fold blunt in the fluorescence signal was observed. No differences were found when extracts were incubated with arginine or xanthine, nor with their respective inhibitors. These data provide evidence that the mitochondrial enzyme succinate dehydrogenase is involved in O_2^- production in response to chronic AGII administration. Also, the absence of significant differences between control mice (untreated) and AGII/rSe-ACE-treated mice indicate the antioxidant capacity of the fraction, being as effective as the control drug at the concentration employed. To determine whether the antioxidant capacity of rSe-ACE is related to the activation of antioxidant enzymes, the activity of GPx, GR, GST and SOD was also evaluated (Fig. S.1). However, none of these enzymes modified its activity after treatment, so the antioxidant effect of rSe-ACE is not through the activation of antioxidant enzymes.

3.4. rSe-ACE prevent vasoconstriction and hypertension

To evaluate whether rSe-ACE controls increased blood pressure, this parameter was evaluated at the beginning (week 0), the middle (week 5), and the end (week 10) of the experiment. Both systolic (SBP) and

diastolic (DBP) blood pressure gradually increased in mice chronically treated with AGII (Fig. 2). At week 5, SBP increased by 30.8% and DBP increased by 13%; by week 10, statistically significant ($P < 0.05$) increases of 46.6% in SBP and 73.1% in DBP were observed with respect to controls. It is noteworthy that no significant increase in blood pressure was observed in AGII/losartan- nor AGII/rSe-ACE-treated mice with respect to controls. This result shows that rSe-ACE is as effective as the pharmacological treatment to control hypertension.

3.5. rSe-ACE prevents inflammation

Considering that O_2^- production promotes inflammation, the anti-inflammatory capability of rSe-ACE in the kidney was evaluated. Kidneys were obtained after the last measure of blood pressure. Cytokine concentrations were evaluated by ELISA in tissue extracts, and ICAM-1 expression was assessed by immunohistochemistry. As shown in Fig. 3, AGII-treated mice showed a significant increase in the levels of IL1 β (21.7%), TNF α (26.7%), IL17A (30.6%), IL4 (19.6%), IL10 (24.7%), and TGF β (35.9%), while no differences were observed in IL6 nor IFN γ . In contrast, no significant differences were found in AGII/losartan- nor AGII/rSe-ACE-administered mice with respect to control mice. ICAM-1 expression in kidneys is shown in Fig. S2. Higher ICAM-1 expression was observed in renal capsule cells (panel B), renal interstitial cells (panel F), and perirenal fat tissue (panel J) in kidneys from AGII-treated mice. Also, an inflammatory infiltrate was observed in the renal capsule and perirenal fat tissue (panels B and J). In contrast, ICAM-1 expression in all evaluated kidney regions was prevented in AGII/rSe-ACE-treated mice. It is remarkable that rSe-ACE had a higher anti-inflammatory effect than losartan, because a moderate expression of ICAM-1 was observed in the kidney regions evaluated, particularly in the capsule and perirenal fat, from AGII/losartan-treated mice.

3.6. rSe-ACE prevents vascular remodeling

A chronicle vascular prooxidant status leads to vascular remodeling. To assess the capacity of rSe-ACE to control AGII-induced vascular remodeling, total and luminal cross section areas of the branch of hepatic artery (BHA) of the portal triad in liver slides were measured in control mice in and AGII-, AGII/rSe-ACE-, and AGII/losartan-treated mice (Fig. 4A–D). The key parameters to assess vascular remodeling were lumen percentage, media thickness percentage, and media/lumen ratio (Fig. 4H–J). Media thickness was calculated by subtracting luminal BHA area from the total BHA. As shown, neither total BHA vascular area, lumen area, nor media thickness (Fig. 4E, F and G, respectively) were

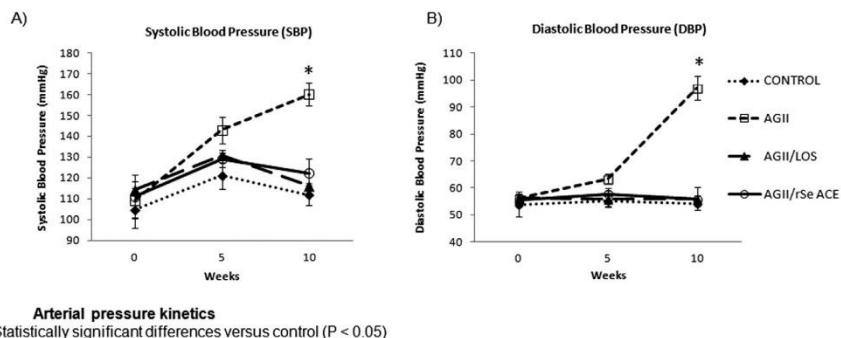
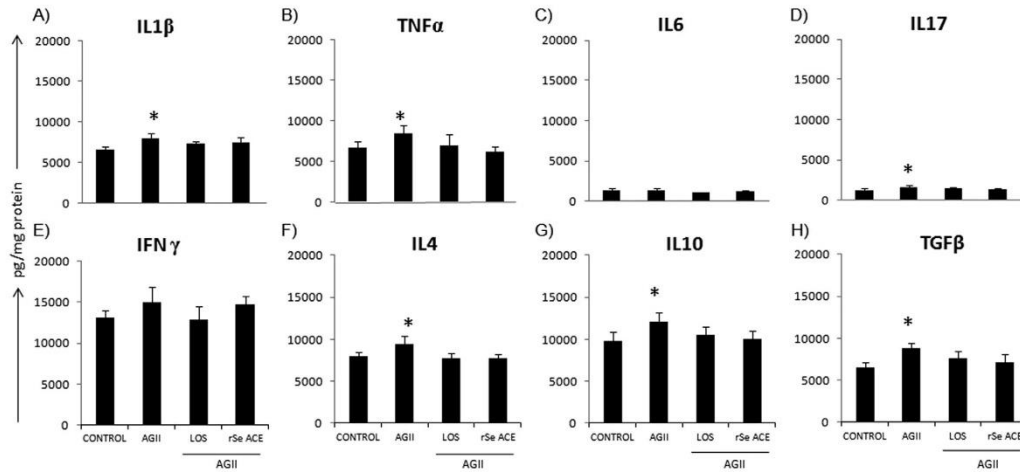


Fig. 2. Arterial pressure kinetics. Systolic and diastolic arterial pressure was measured in mice at weeks 0, 5, and 10. The AGII-treated group showed increases in both pressure readings at week 10, while the rSe-ACE-group maintained pressure values at the levels of the control group. Data are expressed as mean \pm SD and were analyzed by ANOVA and a post-hoc Tukey test. *Statistically significant differences with respect to control ($P < 0.05$). Data are representative of at least three independent experiments.



Kidney cytokine concentration.

*Statistically significant differences versus control ($P < 0.05$).

Fig. 3. Kidney cytokine concentration. Cytokine levels were determined in kidney aqueous extract 10 weeks after AGII administration and treatments. Six kidneys were analyzed per treatment. The control group was treated with saline solution only; AGII 0.1 $\mu\text{g}/\text{kg}/\text{day}$; losartan 10 mg/kg , and rSe-ACE 10 mg/kg . Data are expressed as mean \pm SD and were analyzed by ANOVA-Tukey test. *Statistically significant differences with respect to control ($P < 0.05$).

affected by AGII (Fig. 4B) nor AGII/losartan (Fig. 4C) treatment. In contrast, AGII/rSe-ACE treated mice showed a significant increase ($P < 0.05$) in vascular area (2.3-fold) and lumen area (2.4-fold) (Fig. 4E and F, respectively) but not in media thickness ($P > 0.05$) (Fig. 4G). AGII- and AGII/losartan-treated mice exhibited a significant decrease ($P < 0.05$) in lumen percentage with respect to control mice (2-fold) (Fig. 4H) and a significant increase ($P < 0.05$) (about 1.19-fold) in media thickness and media/lumen ratio (2.7-fold) (Fig. 4I and J, respectively), indicative of hypertrophic vascular remodeling (Intengan and Schiffrin, 2001), a condition present in severe hypertension conditions, such as human secondary hypertension (Rajj et al., 1984). On the other hand, as shown in Fig. 4D, treatment with rSe-ACE prevented this status, since lumen percentage, media thickness, and media/lumen ratio showed no significant differences (Fig. 4 H, I, and J, respectively) with respect to the control group, preventing vascular remodeling.

3.7. rSe-ACE prevents tissue damage

To assess the capacity of rSe-ACE to control tissue damage due to AGII-induced ED, histopathological studies were performed with Masson trichrome stain in liver (Fig. 5) and kidney (Fig. 6), and PAS stain in kidney to evaluate glomerular status (Fig. 7).

3.7.1. rSe-ACE prevents non-alcoholic steatohepatitis (NASH)

In liver, three main pathologic alterations were found in AGII-treated mice: 1) steatosis with perivascular pattern, which originates from the central vein of the hepatic lobule (Fig. 5B); 2) thickening of the trabeculae (Fig. 5F) and of the Glisson's capsule (Fig. 5N and R); and 3) lymphocytic microabscesses with necrosis at the center (Fig. 5J). All these alterations, steatohepatitis, inflammation, and fibrosis are pathological characteristics of NASH. The thickening of trabeculae and Glisson's capsule are due to fiber deposition and the infiltration of mononuclear cells such as lymphocytes, macrophages, fibroblasts, and fibrocytes (Fig. 5N), which indicates the chronicity of the inflammatory

event and the ongoing repairing process. No improvement was observed in the AGII/losartan-treated group with respect to AGII-treated mice (Fig. 5C, G, K, O and S). In contrast, a reduction in AGII-induced steatosis, fiber deposition, cell infiltration to parenchyma (Fig. 5D, H and L, respectively) and Glisson's capsule (Fig. 5 P, T) was observed in rSe-ACE-treated mice.

3.7.2. rSe-ACE prevents glomerulosclerosis

Glomerulosclerosis is a condition often induced by ED (Anderson et al., 1993; Orth et al., 1994), thus, AGII-induced renal damage was evaluated in all mice groups by examining Masson trichrome-stained perirenal fat (Fig. 6A-D), capsules (Fig. 6E-H), perivascular connective tissue (Fig. 6I-L), and cortical glomeruli (Fig. 6M-P). Additionally, the type of infiltrating cells in the capsule (Fig. 6 S) was determined by PAS staining, and a morphometric analysis of the glomerulus was performed (Fig. 7).

As shown in Fig. 6, the AGII-treated group exhibited renal damage characterized by renal capsule thickening (panel F) due to edema, fiber deposition and infiltration of mononuclear cells (mainly lymphocytes and macrophages), characteristic of chronic kidney diseases (CKD) like glomerulosclerosis (Karanovic et al., 2016). Additionally, perivascular fibrosis (panel J), congestion of the tubulointerstitial zone (panel N), and points of mononuclear infiltration (mainly lymphocytes) among perirenal fat adipocytes (panel B) were observed. As shown in the figure, a decrease in the inflammatory process in perirenal fat and renal capsule (panels C and G, respectively) was observed in the losartan-treated mice, although this treatment did not modify the damage to perivascular connective tissue nor the congestion surrounding cortical glomeruli (panel O) induced by AGII (panel K). The rSe-ACE fraction controlled the thickening in perirenal fat and renal capsule (panels D and H, respectively); remarkably, it also diminished the amount of perivascular connective tissue (panel L), preventing the congestion surrounding cortical glomeruli (panel P). These parameters were similar in AGII-rSe-ACE-treated mice and control animals (panels 1 and

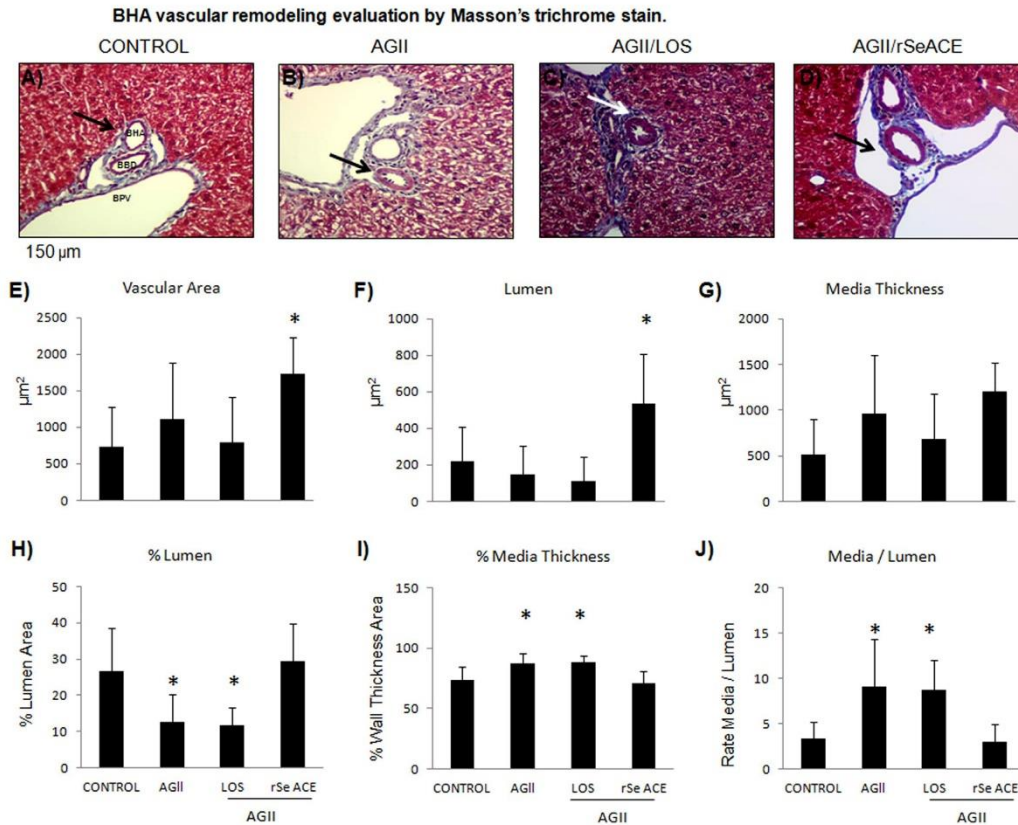


Fig. 4. BHA vascular remodeling evaluation by Masson trichrome stain. A–D) Mouse hepatic portal triad after treatments. E) BHA total area. F) BHA lumen area. G) Thickness of BHA tunica media. H) Lumen and tunica media. I) Percentage with respect to the BHA total area. J) Media area/lumen area ratio. rSe ACE prevented, on one hand, the increase in media thickness percentage, and on the other the decrease in lumen area with respect to the total area and maintained the media/lumen ratio with no significant differences with respect to controls; this ratio was significantly increased in AGII-treated mice. 40 × microphotographs. Branch of portal vein (BPV); branch of hepatic artery (BHA); branch of biliary duct (BBD). Data are expressed as mean ± SD and were analyzed by ANOVA and a post-hoc Tukey-Kramer test. *Statistically significant differences with respect to control ($P < 0.05$).

M, respectively).

Mice treated with AGII exhibited renal capsule thickening, which may be associated to the infiltration of inflammatory cells (mainly lymphocytes, macrophages, and plasmatic cells, and in a lesser extend basophiles and neutrophils) (Fig. S.3B). In losartan-treated mice, infiltration of lymphocytes, macrophages, and fibroblasts was observed (Fig. S3C). On the other hand, infiltration of inflammatory cells was substantially diminished in mice treated with the rSe-ACE fraction (Fig. S.3D).

Glomerulosclerosis, characterized by an increase in glomerular area (37.0%, Fig. 7E), reduced vascular area (52.1%, Fig. 7F), and increased mesangial area (16.1%, Fig. 7G) was clearly found in AGII-treated mice. In the losartan-treated group, vascular area was significantly reduced (27.4%, Fig. 7F) and mesangial area was significantly increased (8.5%, Fig. 7G), even though glomerular area (Fig. 7E) did not exhibit significant differences with respect to controls. In contrast, although the rSe-ACE fraction administration failed to prevent the AGII-induced increase in glomerular area (Fig. 7E), vascular and mesangial areas did not show significant differences (Fig. 7F and G, respectively) with

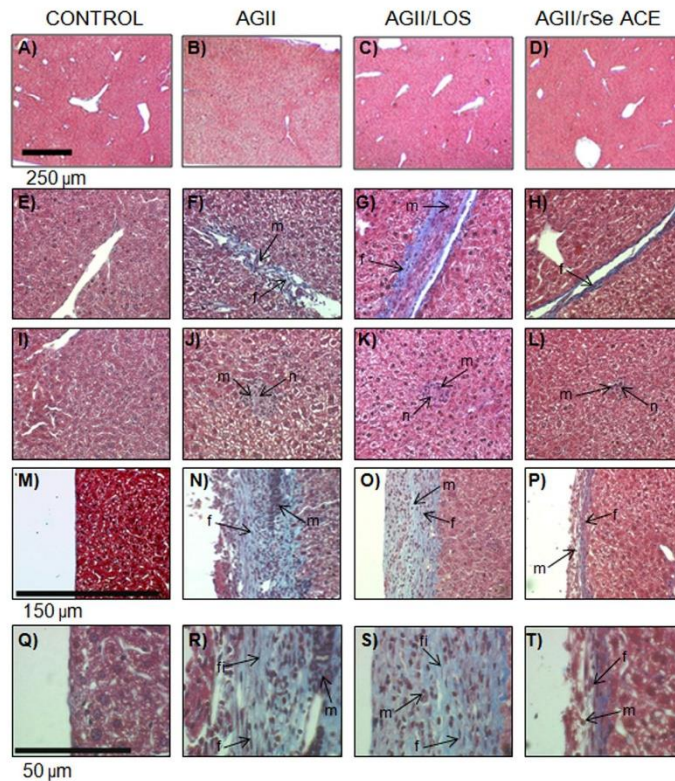
respect to controls. These results imply that although glomeruli are 24.1 times larger than in control mice, the proportion between mesangial and vascular area is conserved, which means that kidney should be working properly, and that the fraction was more efficient than losartan in preventing glomerulosclerosis.

As shown in Fig. 7B, AGII also induces glomerulonephritis, evident in mesangial matrix (Fig. 7G) and glomerulus vascular region hypertrophy (Fig. 7F). The rSe-ACE fraction was able to prevent glomerulonephritis even better than losartan, a finding that points to its effectiveness in preventing renal damage associated to AGII-induced ED.

4. Discussion

This study provides clear evidences of the efficacy of rSe-ACE to prevent ED (and its associated pathologies) induced by chronic AGII administration.

Previous studies showed that both rSe-HA and losartan were able to interfere with the binding of AGII with its receptor, preventing its hypertensive effects (Lombardo-Earl et al., 2014). The rSe-ACE fraction,



Hepatic damage evaluation by Masson's trichrome stain. A-D) Hepatic parenchyma 4X. E-H) trabeculae. J-L) Microabscess. M-P) Glisson's capsule 10X. Q-T) Glisson's capsule 40X

Fig. 5. Hepatic damage evaluation by Masson trichrome stain. A-D) Hepatic parenchyma 4 × . E-H) Trabeculae. J-L) Microabscess. M-P) Glisson's capsule 10 × . Q-T) Glisson's capsule 40 × . AGII-treated group showed steatosis, mononuclear cell infiltrate, fiber deposits and thickening, along with lymphocytic microabscesses, being AGII- and AGII/losartan-treated mice the most severely affected. rSe-ACE prevented severe liver damage. 4 × (bar = 250 µm), 40 × (bar = 150 µm) and 100 × (bar = 50 µm) microphotographs. m: inflammatory mononuclear cells; f: fibroblasts; fi: fibrocytes; n: necrosis.

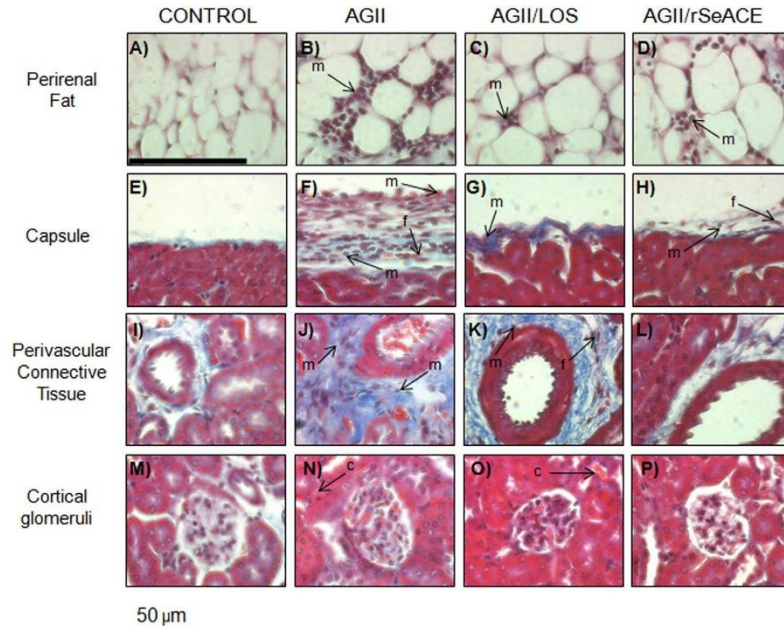
derived from the rSe-HA extract, was found to be enriched in cinnamic acid methyl ester, which could be responsible for the antihypertensive effects in an acute, AGII-induced hypertension model. In this study, the effects of orally administered rSe-ACE on ED pathologies induced by chronic administration of AGII in mice were further characterized.

In this model, ED is induced by AGII binding to the AT1R receptor on endothelial cells, which activates NAD(P)H oxidase through PKC, inducing the production of ROS (O_2^-) and leading to a prooxidative status. This reduces the bioavailability of nitric oxide (NO), causing vasoconstriction. PKC activates the NFκB transcription factor and the production of proinflammatory cytokines (Jaimes et al., 2010; Marchesi et al., 2008; Nishida et al., 2010; Schramm et al., 2012), which ultimately lead to vascular remodeling and tissue damage. As demonstrated in our study, AGII-treated mice exhibited higher levels of inflammatory and non-inflammatory cytokines in kidney (Fig. 3), a target tissue for ED (Bosch et al., 2010; Henke et al., 2007; Rajendran et al., 2013); inflammatory cytokines in ED could be produced not only by endothelial cells and VSMC, but also by artery-associated cells such as neutrophils, mastocytes, and lymphocytes (Sprague and Khalil, 2009). As shown in Fig. S.3, these cells were recruited to the inflamed kidney

by AGII administration, where they mediate a proinflammatory, prooxidative status leading to vascular remodeling and damage (Brazin et al., 2014; Guasti et al., 2008; Hallersund et al., 2011).

The rSe-ACE extract effectively controlled the prooxidant and proinflammatory status, as well as vasoconstriction, vascular remodeling, and tissue damage, despite the concomitant administration of AGII. This protective effect was higher than that of losartan, since the latter only controlled oxidative stress, hypertension, and the production of proinflammatory cytokines, but not ICAM-1 expression, vascular remodeling, tissue injury, proinflammatory cell infiltrate, nor glomerular damage.

Cinnamic acid was identified as the main component of rSe-ACE along with other less polar fatty acids, specifically palmitic, linoleic, linoleic acid chloride, 11-Z-hexadecenoic acid, 2-palmitoylglycerol, and linolenic acid methyl ester (Fig. 1, Table 1). These compounds have been reported to show anti-inflammatory properties (Bartolome et al., 2013). Cinnamic acid can reduce the concentration of ROS (O_2^-) by two different mechanisms: by reacting with the radicals through its electron-rich double bonds (Azzu and Brand, 2010), acting thus as a scavenger, and by preventing ROS production by mitochondrial



Renal damage evaluation by Masson's trichrome stain. A-D) Perirenal fat tissue. E-H) Renal capsule. I-L) Perivascular connective tissue. M-P) Cortical glomerulus.

Fig. 6. Renal damage evaluation by Masson's trichrome stain. A-D) Perirenal fat tissue. E-H) Renal capsule. I-L) Perivascular connective tissue. M-P) Cortical glomerulus. Inflammatory damage due to edema and the presence of mononuclear cells and fibroblasts is observed in fat tissue, capsule and perivascular tissue, being AGII-treated mice the most severely affected. rSe-ACE ameliorated these damage patterns with higher efficiency than losartan. 100 × microphotographs. m: mononuclear cells; f: fibroblasts; c: congestion.

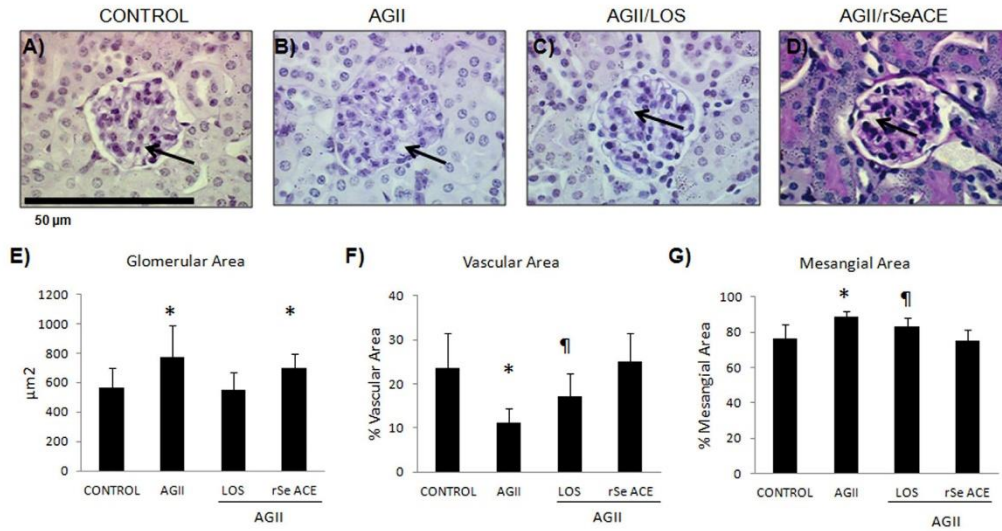
enzymes like succinate dehydrogenase, since cinnamic acid was demonstrated to activate uncoupled proteins (UCP) via its 2-alkenal group (Echtay et al., 2003). UCP are a subfamily of mitochondrial proteins that decrease ROS production by uncoupling mitochondrial enzymes, thus preventing oxidative damage (Azzu and Brand, 2010; Brand et al., 2004). In our study, rSe-ACE was found to reduce ROS production by succinate dehydrogenase, probably due to its high content of cinnamic acid.

The role of growth factors like TGFβ in ED has been related to extracellular matrix deposition, promoting VSMC hypertrophy and hyperplasia, leading to vascular remodeling and fibrosis (Gibbons et al., 1992; Ruiz-Ortega et al., 2006). The latter are key factors in the pathogenesis of renal and hepatic diseases and were observed in hepatic tissues from AGII-treated mice (Figs. 4 and 5) but not in renal tissues (Figs. 6 and 7). AGII administration also induces the expression of adhesion molecules like E- and P-selectins, VCAM-1, and ICAM-1 (Chen et al., 2015; da Cunha et al., 2005; Liang et al., 2015), which along with the proinflammatory environment would favor the recruitment and activation of monocytes, which could migrate to the media and adventitia layers of the blood vessels, releasing effector molecules and further contributing to tissue damage (Marchesi et al., 2008; Nishida et al., 2010; Touyz, 2004). In this study, AGII increased ICAM-1 expression in the renal capsule, renal interstice, and perirenal fat; this effect was extensively prevented in rSe-ACE-treated mice, while only a reduction in ICAM-1 expression was attained in losartan-treated mice. The effects of rSe-ACE could be mediated by cinnamic acid and other

less polar fatty acids, which individually have been proved to exert anti-inflammatory and antioxidant effects (Kersten et al., 2000; Nunn et al., 2007), acting synergistically to prevent tissue damage.

It has been reported that the inability of the endothelium to couple hemodynamic events efficiently is related to the low bioavailability of nitric oxide (NO). This molecule acts as a second messenger and its absence contributes to hypertension and abnormal vascular remodeling (Rudic and Sessa, 1999). Treatment with AGII induced hypertension in mice comparable with grades 2 and 3 of the human disease (Mansia et al., 2007; Savoia et al., 2011). Considering that rSe-ACE was able to control the increase in ROS, it is possible that this treatment increased NO bioavailability, regulating hypertension (the main pathology resulting from ED) and vascular remodeling. Moreover, rSe-ACE not only prevented vascular remodeling but also induced a compensatory enlargement in the branch of hepatic portal vein and in the lumen of blood vessels, without increasing the thickness of the media and maintaining the relation media/lumen with no statistically significant differences with the control group. This represents an adaptive response to preserve the normal blood flow and could be related with NO availability mediated by the antioxidant activity of the fraction, as discussed above.

Another point that merits comments is the capability of the rSe-ACE fraction to control non-alcoholic steatohepatitis (NASH) induced by the chronic administration of AGII (Fig. 5). NASH progression is characterized by fatty liver, damage to hepatocytes, fibrosis, and inflammation (Brunt, 2004; de Alwis and Day, 2008). The



Renal damage evaluation by PAS stain. A-D) glomeruli. E-G) Glomerular area and glomerular vascular and mesangial area percent age. *Statistically significant differences versus control ($P < 0.05$). † Statistically significant differences versus control and AGII.

Fig. 7. Renal damage evaluation by PAS stain. A-D) Glomeruli. E-G) Glomerular area and glomerular vascular and mesangial area percentage. Glomeruli from AGII- and AGII/losartan-treated mice (B and C) showed expansion of mesangial cells and collapse of glomerular vasculature. rSe-ACE (D-G) restored glomerular vasculature to the status of the control group. Arrows indicate vascular areas. 100 × microphotographs. Data are expressed as mean ± SD and were analyzed by ANOVA and a post-hoc Tukey test. *Statistically significant differences with respect to control ($P < 0.05$). † Statistically significant differences with respect to control and AGII.

pathophysiological hallmark of NASH and non-alcoholic fatty liver disease (NAFLD) is insulin resistance (IR), which increases the flux of free fatty acids from the adipose tissue to non-adipose organs, resulting in an abnormal fat metabolism that leads to an accumulation of hepatic triglycerides (Bugianesi et al., 2005). AGII exerts its action on insulin-sensitive tissues such as liver, where it has effects on insulin receptors (Morris et al., 2013; Olivares-Reyes et al., 2009; Togashi et al., 2000), favoring lipid accumulation in hepatocytes and impairing the oxidative capacity of the mitochondria, leading to an increased ROS generation and its consequences (Rolo et al., 2012). rSe-ACE was capable of controlling NASH, probably through the synergistic action of cinnamic acid and other less polar fatty acids. Previous studies have shown that cinnamic acid is not only able to control the production of ROS, which play an important role in NASH induction, but it also alleviates insulin resistance *in vivo* and *in vitro* (Huang et al., 2009; Huang and Shen, 2012; Lakshmi et al., 2009). On the other hand, other less polar fatty acids in the rSe-ACE fraction probably contributed to control the inflammatory status.

It is well known that AGII, via AT1R, participates in the pathogenesis of renal diseases through the increased production of ROS (for instance, by promoting hyperplasia/hypertrophy in several cell types; upregulating the expression of cytokines, cell adhesion molecules and TGFβ). All these alterations would increase the synthesis of extracellular matrix proteins through the activation of mesangial cells and interstitial and tubular fibroblasts, the stimulation of plasminogen activator inhibitor-1 (PAI-1) and the infiltration and activation of macrophages, which would further increase inflammation, fibrosis, and ROS production (Mezzano et al., 2001; Ruiz-Ortega et al., 2001, 2006). In this study, AGII-treated mice developed structural alterations like

edema, deposit of collagen fibers and infiltration of mononuclear cells (lymphocytes, mastocytes, neutrophils, and macrophages), all of which were controlled by the administration of rSe-ACE.

Finally, our results indicate that AGII administration induced glomerulosclerosis (a chronic kidney disease) by increasing mesangial volume (Anderson et al., 1993; Orth et al., 1994) (Fig. 7). AGII has been reported to activate mesangial and tubular cells and interstitial fibroblasts, increasing the expression and synthesis of extracellular matrix proteins mediated by the release of TGFβ; AGII also participates in the inflammatory response, inducing the activation of mononuclear cells and increasing the levels of proinflammatory mediators (Mezzano et al., 2001), conditions that were observed in AGII-treated mice. TGFβ is thought to be fibrogenic, either directly or indirectly, through the induction of connective tissue growth factor (Meguid et al., 2005; Ruiz-Ortega et al., 2001, 2006). Recent studies demonstrated that the activation of Nrf2 protects various tissues and organs against fibrosis via inhibition of TGF/Smad signaling (Oh et al., 2012). Nrf2 is a transcription factor that regulates the expression of genes encoding phase-2 enzymes (heme oxygenase-1, glutathione-S-transferase A1, and NAD(P)H:quinone oxidoreductase 1) (Korkina, 2007), which can counteract inflammatory, fibrinogenic, and oxidative damages. There is considerable experimental evidence suggesting that Nrf2 signaling plays a protective role in renal injuries caused by various pathologic conditions (Choi et al., 2009, 2014). The cinnamic acid present in rSe-ACE has been reported to induce Nrf2 dissociation from Keap 1, leading to Nrf2 translocation to the nucleus, favoring the expression of ARE-regulated cytoprotective genes (Korkina, 2007); therefore, it is possible that some of the effects observed by the rSe-ACE extract could be mediated by these actions of cinnamic acid.

Altogether, these results show that rSe-ACE could control the wide range of effects of AGII and could be used as a comprehensive treatment for ED, being probably one of the most promissory approaches to prevent fibrosis in progressive renal diseases.

5. Conclusion

The results reported in this study indicate that rSe-ACE is able to control ED and associated pathologies such as hypertension, renal and liver damage, being a good candidate for the development of a phytochemical medicine for ED, avoiding the need for polydrug treatments, frequently required to control these pathologies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2018.02.036>.

References

Adya, R., Tan, B.K., Randeve, H.S., 2015. Differential effects of leptin and adiponectin in endothelial angiogenesis. *J. Diabetes Res.* 2015, 648239.

Anderon, P.W., Do, Y.S., Hsueh, W.A., 1993. Angiotensin II causes mesangial cell hypertrophy. *Hypertension* 21 (1), 29–35.

Argueta, V.A., Cano, A.L., Rodarte, M.E., 1994. Atlas de las plantas de la medicina Tradicional mexicana. Inst. Nac. Indig.; México 1994 (1), 393–394.

Azza, V., Brand, M.D., 2010. The on-off switches of the mitochondrial uncoupling proteins. *Trends Biochem. Sci.* 35 (5), 298–307.

Bartolome, A.P., Villaseñor, I.M., Yang, W.C., 2013. *Bidens pilosa* L. (Asteraceae): botanical properties, traditional uses, phytochemistry, and pharmacology. *Evid. Based Complement. Altern. Med.* 2013, 340215.

Bastaki, S.M.A., Padol, I.T., Amir, N., Hunt, R.H., 2017. Effect of Aspirin and ibuprofen either alone or in combination on gastric mucosa and bleeding time and on serum prostaglandin E2 and thromboxane A2 levels in the anaesthetized rats *in vivo*. *Mol. Cell. Biochem.* <http://dx.doi.org/10.1007/s11010-017-3110-1>.

Bosch, J., Abraides, J.G., Fernández, M., García-Pagán, J.C., 2010. Hepatic endothelial

dysfunction and abnormal angiogenesis: new targets in the treatment of portal hypertension. *J. Hepatol.* 53 (3), 558–567.

Brand, M.D., Affourtit, C., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Pakay, J.L., Parker, N., 2004. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic. Biol. Med.* 37 (6), 755–767.

Brazin, J., Malliaris, S., Groh, B., Mehra, B., Hidalgo, D., Otterburn, D., Silver, R.B., Spector, J.A., 2014. Mast cells in the periprosthetic breast capsule. *Aesthetic Plast. Surg.* 38 (3), 592–601.

Brunt, E.M., 2004. Nonalcoholic steatohepatitis. *Semin. Liver Dis.* 24 (1), 3–20.

Bugianesi, E., McCullough, A.J., Marchesini, G., 2005. Insulin resistance: a metabolic pathway to chronic liver disease. *Hepatology* 42 (5), 987–1000.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* 250, 5475–5480.

Chen, W.D., Chu, Y.F., Li, X.D., Gao, P.J., 2015. Angiotensin II induces expression of inflammatory mediators in vascular adventitial fibroblasts. *Sheng Li Xue Bao.* 25 (6), 603–610 (6).

Choi, H.K., Pokharel, Y.R., Lim, S.C., Han, H.K., Ryu, C.S., Kim, S., Kwak, M.K., Kang, K.W., 2009. Inhibition of liver fibrosis by solubilized coenzyme Q10: role of Nrf2 activation in inhibiting transforming growth factor- β 1 expression. *Toxicol. Appl. Pharmacol.* 240 (3), 377–384.

Choi, B.H., Kang, K.S., Kwak, M.K., 2014. Effect of redox modulating NRF2 activators on chronic kidney disease. *Molecules* 19 (8), 12727–12759.

da Cunha, V., Tham, D.M., Martin-McNulty, B., Deng, G., Ho, J.J., Wilson, D.W., Rutledge, J.C., Vergona, R., Sullivan, M.E., Wang, Y.X., 2005. Enalapril attenuates angiotensin II-induced atherosclerosis and vascular inflammation. *Atherosclerosis* 178 (1), 9–17.

de Alwis, N.M.W., Day, C.P., 2008. Non-alcoholic fatty liver disease: the mist gradually clears. *J. Hepatol.* 48 (Suppl 1), S104–S112.

Echtay, K.S., Esteves, T.C., Pakay, J.L., Jakabsons, M.B., Lambert, A.J., Portero-Otin, M., Pamplona, R., Vidal-Puig, A.J., Wang, S., Roebuck, S.J., Brand, M.D., 2003. A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *EMBO J.* 22 (16), 4103–4110.

Endemann, D.H., Schiffrin, E.L., 2004. Endothelial dysfunction. *J. Am. Soc. Nephrol.* 15 (8), 1983–1992.

Feldman, D.L., Christian, J.J., 1981. The development of an acth-induced renal glomerular lesion in *Mus musculus*. An ultrastructural study. *J. Pathol.* 134 (3), 181–197.

Gérard-Monnier, D., Erdelmeier, L., Régnard, K., Moze-Henry, N., Yadan, J.C., Chaudière, J., 1998. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chem. Res. Toxicol.* 11, 1176–1183.

Gibbons, G.H., Pratt, R.E., Dzau, V.J., 1992. Vascular smooth muscle cell hypertrophy vs. hyperplasia. Autocrine transforming growth factor- β 1 expression determines growth response to angiotensin II. *J. Clin. Investig.* 90 (2), 456–461.

Goldner, J., 1938. A modification of the Masson trichrome technique for routine laboratory purposes. *Am. J. Pathol.* 14 (2), 237.

Gorman, J.L., Liu, S.T., Slopick, D., Shariati, K., Hasamee, A., Olenich, S., Olfert, L.M., Haas, T.L., 2014. Angiotensin II evokes angiogenic signals within skeletal muscle through co-ordinated effects on skeletal myocytes and endothelial cells (9). *PLoS One* 9 (1), e85537. <http://dx.doi.org/10.1371/journal.pone.0085537>.

Guastrini, L., Marino, F., Cosentino, M., Maio, R.C., Rasini, E., Ferrari, M., Castiglioni, L., Klersy, C., Gaudio, G., Grandi, A.M., Lecchini, S., Venco, A., 2008. Prolonged statin-associated reduction in neutrophil reactive oxygen species and angiotensin II type 1 receptor expression: 1-year follow-up. *Eur. Heart J.* 29 (9), 1118–1126.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.

Hallersund, P., Elfvin, A., Helander, H.F., Fändriks, L., 2011. The expression of renin-angiotensin system components in the human gastric mucosa. *J. Renin Angiotensin Aldosterone Syst.* 12 (1), 54–64.

Henke, N., Schmidt-Ullrich, R., Dechend, R., Park, J.K., Qadri, F., Wellner, M., Obst, M., Gross, V., Dietz, R., Luft, F.C., Scheidereit, C., Müller, D.N., 2007. Vascular endothelial cell-specific NF- κ B suppression attenuates hypertension-induced renal damage. *Circ. Res.* 3 (101), 268–276 (3).

Huang, D.W., Shen, S.C., Wu, J.S.B., 2009. Effects of caffeic acid and cinnamic acid on glucose uptake in insulin-resistant mouse hepatocytes. *J. Agric. Food Chem.* 57 (17), 7687–7692.

Huang, D.W., Shen, S.C., 2012. Caffeic acid and cinnamic acid ameliorate glucose metabolism via modulating glycogenesis and gluconeogenesis in insulin-resistant mouse hepatocytes. *J. Funct. Foods* 4 (1), 358–366. <http://dx.doi.org/10.1016/j.jff.2012.01.005>.

Husain, K., Hernandez, W., Ansari, R.A., Ferder, L., 2015. Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J. Biol. Chem.* 26 (6), 209–217.

Ibarra-Alvarado, C., Rojas, A., Mendoza, S., Bah, M., Gutiérrez, D.M., Hernández-Sandoval, I., Martínez, M., 2010. Vasoactive and antioxidant activities of plants used in Mexican traditional medicine for the treatment of cardiovascular diseases. *Pharm. Biol.* 48 (7), 732–739. <http://dx.doi.org/10.3109/13880200903271280>.

Intengan, H.D., Schiffrin, E.L., 2001. Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. *Hypertension* 38, 581–587.

Jaimes, E.A., Hua, P., Tian, R.X., Raji, L., 2010. Human glomerular endothelium: interplay among glucose, free fatty acids, angiotensin II, and oxidative stress. *Am. J. Physiol. Ren. Physiol.* 298, F125–F132. <http://dx.doi.org/10.1152/ajprenal.00248.2009>.

Karanovic, D., Grujic-Milanovic, J., Miloradovic, Z., Ivanov, M., Jovovic, D., Vajic, U.J., Zivotic, M., Markovic-Lipkovic, J., Mihailovic-Stanojevic, N., 2016. Effects of single and combined losartan and tempol treatments on oxidative stress, kidney structure

- and function in spontaneously hypertensive rats with early course of proteinuric nephropathy. *PLoS One* 25 (11), e0161706 (8).
- Kersten, S., Desvergne, B., Wahli, W., 2000. Roles of PPARs in health and disease. *Nature* 25 (405), 421–424 (6785).
- Korkina, L.G., 2007. Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cell. Mol. Biol.* 53 (1), 15–25.
- Lakshmi, B.S., Sujatha, S., Anand, S., Sangeetha, K.N., Narayanan, R.B., Katiyar, C., Kanaujia, A., Duggar, R., Singh, Y., Srinivas, K., Bansal, V., Sarin, S., Tandon, R., Sharma, S., Singh, S., 2009. Cinnamic acid, from the bark of *Cinnamomum cassia*, regulates glucose transport via activation of GLUT4 on L6 myotubes in a phosphatidylinositol 3-kinase-independent manner. *J. Diabetes* 1 (2), 99–106.
- Lawrence, R.A., Burk, R.F., 1976. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem. Biophys. Res. Commun.* 23 (71), 952–958 (4).
- Liang, B., Wang, X., Zhang, N., Yang, H., Bai, R., Liu, M., Bian, Y., Xiao, C., Yang, Z., 2015. Angiotensin-(1-7) Attenuates Angiotensin II-Induced ICAM-1, VCAM-1, and MCP-1 Expression via the MAS Receptor Through Suppression of P38 and NF- κ B Pathways in HUVECs. *Cell Physiol. Biochem.* 35 (6), 2472–2482.
- Liu, H., Chen, T., Li, N., Wang, S., Bu, P., 2015. Role of SIRT3 in Angiotensin II-Induced human umbilical vein endothelial cells dysfunction. *BMC Cardiovasc. Disord.* 30, 15–81.
- Lombardo-Earl, G., Roman-Ramos, R., Zamila, A., Herrera-Ruiz, M., Rosas-Salgado, G., Tortoriello, J., Jiménez-Ferrer, E., 2014. Extracts and fractions from edible roots of *Sechium edule* (Jacq.) Sw. with antihypertensive activity. *Evid. Based Complement Altern. Med.* 2014, 594326. <http://dx.doi.org/10.1155/2014/594326>.
- Lozoya, X., 1980. Mexican medicinal plants used for treatment of cardiovascular diseases. *Am. J. Chin. Med.* 8 (1–2), 86–95.
- Mansia, G., De Backer, G., Dominiczak, A., Cifkova, R., Fagard, R., Germano, G., Grassi, G., Heagerty, A.M., Kjeldsen, S.E., Laurent, S., Narkiewicz, K., Ruilope, L., Rynkiewicz, A., Schmieder, R.E., Struijker, B.H.A., Zanchetti, A., European Society of Hypertension; European Society of Cardiology, 2007. 2007 ESH-ESC Guidelines for the management of arterial hypertension: the task force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Blood Press.* 16 (3), 135–232.
- Marchesi, C., Paradis, P., Schiffrin, E.L., 2008. Role of the renin-angiotensin system in vascular inflammation. *Trends Pharmacol. Sci.* 29, 367–374.
- Marques, F.Z., Campain, A.E., Davern, P.J., Yang, Y.H., Head, G.A., Morris, B.J., 2011. Genes influencing circadian differences in blood pressure in hypertensive mice. *PLoS One* 6, 1–9. <http://dx.doi.org/10.1371/journal.pone.0019203>.
- Meguid, E.I., Nishas, A., Bello, A.K., 2005. Chronic kidney disease: the global challenge. *Lancet* 22–28 (365), 331–340 (9456).
- Mezzano, S.A., Ruiz-Ortega, M., Egido, J., 2001. Angiotensin II and renal fibrosis. *Hypertension* 38, 635–638.
- Morris, E.M., Fletcher, J.A., Thyfault, J.P., Rector, R.S., 2013. The role of angiotensin II in nonalcoholic steatohepatitis. *Mol. Cell Endocrinol.* 378 (1), 29–40.
- Nishida, M., Kitajima, N., Saito, S., Nakaya, M., Kurose, H., 2010. Regulation of Angiotensin II receptor signaling by cysteine modification of NF- κ B. *Nitric Oxide* 25, 112–117.
- Nunn, A.V., Bell, J., Barter, P., 2007. The integration of lipid-sensing and anti-inflammatory effects: how the PPARs play a role in metabolic balance. *Nucl. Recept.* 25. <http://dx.doi.org/10.1186/1478-1336-5-1>. 5–1.
- Oberley, L.W., Spitz, D.R., 1984. Assay of superoxide dismutase activity in tumor tissue. *Methods Enzymol.* 105, 457–464.
- Oh, C.J., Kim, J.Y., Min, A.K., Park, K.G., Harris, R.A., Kim, H.J., Lee, I.K., 2012. Sulforaphane attenuates hepatic fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-beta/Smad signaling. *Free Radic. Biol. Med.* 52, 671–682.
- Olivares-Reyes, J.A., Arellano-Plancarte, A., Castillo-Hernandez, J.R., 2009. Angiotensin II and the development of insulin resistance: implications for diabetes. *Mol. Cell. Endocrinol.* 302 (2), 128–139.
- Orth, S.R., Weinreich, T., Bönisch, S., Weih, M., Ritz, E., 1994. Angiotensin II induces hypertrophy and hyperplasia in adult human mesangial cells. *Exp. Nephrol.* 3 (1), 23–33.
- Raij, L., Azar, S., Keane, W., 1984. Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. *Kidney Int.* 26, 137–143.
- Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., Nishigaki, I., 2013. The vascular endothelium and human diseases. *Int. J. Biol. Sci.* 9 (10), 1057–1069.
- Rolo, A.P., Teodoro, J.S., Palmeira, C.M., 2012. Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis. *Free Radic. Biol. Med.* 52 (1), 59–69.
- Rudic, R.D., Sessa, W.C., 1999. Nitric oxide in endothelial dysfunction and vascular remodeling: clinical correlates and experimental links. *Am. J. Hum. Genet.* 64 (3), 673–677.
- Ruiz-Ortega, M., Rupérez, M., Esteban, V., Rodríguez-Vila, J., Sánchez-López, E., Carvajal, G., Egido, J., 2006. Angiotensin II: a key factor in the inflammatory and fibrotic response in kidney diseases. *Nephrol. Dial. Transplant.* 21 (1), 16–20.
- Ruiz-Ortega, M., Lorenzo, O., Suzuki, Y., Rupérez, M., Egido, J., 2001. Pro-inflammatory actions of angiotensins. *Curr. Opin. Nephrol. Hypertens.* 10, 321–329.
- Satoh, M., Fujimoto, S., Haruna, Y., Arakawa, S., Horike, H., Komai, N., Sasaki, T., Tsujioka, K., Makino, H., Kashiwara, N., 2005. NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. *Am. J. Physiol. Ren. Physiol.* 288, F1144–F1152.
- Savoia, C., Sada, L., Zezza, L., Pucci, L., Lauri, F.M., Befani, A., Alonzo, A., Volpe, M., 2011. Vascular inflammation and endothelial dysfunction in experimental hypertension. *Int. J. Hypertens.* 2011, 281240.
- Schmitt, C.A., Dirsch, V.M., 2009. Modulation of endothelial nitric oxide by plant-derived products. *Nitric Oxide* 2, 77–91.
- Schramm, A., Matusik, P., Osmenda, G., Guzik, T.J., 2012. Targeting NADPH oxidases in vascular pharmacology. *Vasc. Pharmacol.* 56 (5–6), 216–231.
- Sprague, A.H., Khalil, R.A., 2009. Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem. Pharmacol.* 15 (78), 539–552 (6).
- The Plant List, 2013. A Working List of All Plant Species** (www.theplantlist.org).
- Togashi, N., Ura, N., Higashiura, K., Murakami, H., Shimamoto, K., 2000. The contribution of skeletal muscle tumor necrosis factor- α to insulin resistance and hypertension in fructose-fed rats. *J. Hypertens.* 18, 1605–1610.
- Touyz, R.M., 2004. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* 44, 248–252.

13. Bibliografía

Abou-Zaid MM, Lombardo DA, Kite GC, Grayer RJ, Veitch NC (2001) Acylated flavone C-glycosides from *Cucumis sativus* . *Phytochemistry* 58(1):167–172).

A. Abdul-Ghani, Devjit Tripathy, Ralph A. Defronzo, Contributions of β -Cell Dysfunction and Insulin Resistance to the Pathogenesis of Impaired Glucose Tolerance and Impaired Fasting Glucose MUHAMMAD, *Diabetes care* 29:1130-1139, 2006.

Ahima, Rexford S. Adipose tissue as an endocrine organ. *Obesity*. 2006;14(Suppl 5):242S–249S.

Alfaro Villanueva J. Optimización multi-respuesta según la velocidad de lanzado, número de operadores, frecuencia de moto reductor, sobre el % de desviación del calibre y % de aprovechamiento final, en el proceso de selección de espárrago blanco fresco en agualima S.A.C- Enero 2014, Tesis para obtener el título de ingeniero estadístico, otorgado por la Universidad Nacional de Trujillo, Perú.

Almanza-Perez JC, Alarcon-Aguilar FJ, Blancas-Flores G, Campos-Sepulveda AE, Roman-Ramos R, Garcia-Macedo R, Cruz M. (2010). Glycine regulates inflammatory markers modifying the energetic balance through PPAR and UCP-2. *Biomed Pharmacother.* ; 64(8):534-40.

Ángeles Fortis-Barrera, Francisco Javier Alarcón-Aguilar, Tania Banderas-Dorantes, Margarita Díaz-Flores, Rubén Román-Ramos, Miguel Cruz and Rebeca García-Macedo. *Cucurbita ficifolia* Bouché (Cucurbitaceae) and D-chiro-inositol modulate the redox state and inflammation in 3T3-L1 adipocytes, 2013, Royal Pharmaceutical Society, *Journal of Pharmacy and Pharmacology*, 65, pp. 1563–1576

Angélica Ruiz-Ramírez, Ely Ortiz-Balderas, Guillermo Cardozo-Saldaña, Eulises Díaz-Díaz, Mohammed El-Hafidi. Glycine restores glutathione and protects against oxidative stress in vascular tissue from sucrose-fed rats *Clinical Science* Aug 15, 2013, 126(1)19-29; DOI: 10.1042/CS20130164

A.R.G. Proença, R.A.L. Sertié, A.C. Oliveira, A.B. Campaña, R.O. Caminhotto P. Chimin and F.B. Lima, New concepts in white adipose tissue physiology. *Brazilian Journal of Medical and Biological Research* (2014) 47(3): 192-205.

Arteaga A., Méndez M., Nuñez P., Trejo C., Mayagoitia L., Ramírez M., Mazón E., Zamilpa A., Tortoriello J., Yamamoto J., Fragoso G., Jiménez E, Rosas G., 2011, Evaluación de 3 extractos de *cucumis sativus* para controlar la metainflamación y las patologías asociadas a la obesidad. XX Congreso nacional de inmunología, 17-21 de abril, 2011. Pag 113 N° de cartel 336.

Blancas-Flores G., Almanza-Pérez J.C., López-Roa R.I., Alarcón-Aguilar F.J., García-Macedo R., Cruz M., 2010. Obesity as an inflammatory process. *Bol Med Hosp Infant Mex.* 67.

Bernardo Léo Wajchenberg, Marcia Nery, Maria Rosaria Cunha, Maria Elizabeth Rossi da Silva, Adipose tissue at the crossroads in the development of the metabolic syndrome, inflammation and atherosclerosis, *Arq Bras Endocrinol Metab.* 2009;53(2):145-150.

Bogdanski P, Szulinska M, Suliburska J, Pupek-Musialik D, Jablecka A, Witmanowski H. Supplementation with L-arginine favorably influences plasminogen activator inhibitor type 1 concentration in obese patients. A randomized, double blind trial. *J Endocrinol Invest* 2013, 36:221–226.

Cachofeiro V., Miana M., Martín-Fernández B., de las Heras N., Lahera V., 2006, Obesidad, inflamación y disfunción endotelial. *Rev Esp Obes*, 4 (4):195-204.

Cipolletta D., Kolodin D., Benoist C. y Mathis D., 2011, Tissue-resident Foxp3+CD4+ T cells that impacts organismal metabolism, *Semin Immunol*.

Chen SF, Xiao XC, Sun YS, Zheng L, Feng X. Effects and mechanisms of resveratrol on proliferation and differentiation of murine 3T3-L1 cells. *Chin Pharmacol Bull* 2010; 26: 108–111

Chen Y, Tian J, Tian X, Tang X, Rui K, et al. (2014) Adipose Tissue Dendritic Cells Enhances Inflammation by Prompting the Generation of Th17 Cells. *PLoS ONE* 9(3): e92450. doi:10.1371/journal.pone.0092450.

Christiane Ott, Kathleen Jacobs, Elisa Haucke, Anne Navarrete Santos, Tilman Grune, Andreas Simm Role of advanced glycation end products in cellular signaling. *Redox Biology* 2(2014)411–429.

De Ferranti S., Mozaffarian D., 2009, La tormenta perfecta: obesidad, disfunción del adipocito y consecuencias metabólicas. *Clin Chem*. 34(2):95-108.

Eun Jung Cho, Xiang Lan Piao, Moon Hee Jang, Seung Hoon Baek, Hyun Young Kim, Ki Sung Kang, Sung Won Kwon, Jeong Hill Park, The effect of steaming on the free amino acid contents and antioxidant activity of *Panax ginseng*. *Food Chemistry* 107 (2008) 876–882.

Fábio Santos Lira, José Cesar Rosa, Gustavo Duarte Pimentel, Marília Seelaender, Ana Raimunda Damaso, Lila Missae Oyama, Claudia Oller do Nascimento, Both adiponectin and interleukin-10 inhibit LPS-induced activation of the NF- κ B pathway in 3T3-L1 adipocytes, *Cytokine* 57 (2012) 98–106.

Fernández-Sánchez A., Madrigal-Santillán E., Bautista M., Esquivel-Soto J., Morales-González A., Esquivel-Chirino C., Durante-Montiel I., Sánchez-Rivera G, Valadez-Vega C. y Morales-González J.A., 2011, Inflammation, Oxidative Stress, and Obesity, *International Journal of Molecular Sciences*, 12:3117-3132.

Freedman D.S., Gillespie C., Park S., 2011, Incidences of obesity and extreme obesity among US adults: findings from the 2009 Behavioral Risk Factor Surveillance System. *Population Health Metrics*. 9:5

Fu WJ, Haynes TE, Kohli R, Hu J, Shi W, Spencer TE, Carroll RJ, Meiningner CJ, and Wu G. Dietary L-Arginine Supplementation Reduces Fat Mass in Zucker Diabetic Fatty Rats, *Nutr*. 2005, 135(4):714-721

Ghule BV, Ghante MH, Saoji AN, Yeole PG. 2009 Antihyperlipidemic effect of the methanolic extract from *Lagenaria siceraria* Stand.fruit in hyperlipidemic rats. *J Ethnopharmacol*. 124(2):333-7.

Gideon R. Hajer, Timon W. Van Haeften and Frank L.J. Visseren. Adipose tissue dysfunction in obesity and vascular disease. *European Heart Journal* (2008) 29, 2959-2971.

Giuseppe Murdolo, Marta Piroddi, Francesca Luchetti, Cristina Tortoioli, Barbara Canonico, Chiara Zerbinati, Francesco Galli, Luigi Iuliano, Oxidative stress and lipid peroxidation by-products at the crossroad between adipose organ dysregulation and obesity-linked insulin resistance, *Biochimie* 95 (2013) 585e594.

Gregor MF, Hotamisligil GS. Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. *J Lipid Res*. 2007; 48: 1905-14. PMID: 17699733 DOI: 10.1194/jlr.R700007-JLR200

Gutiérrez JP, Rivera-Dommarco J, Shamah-Levy T, Villalpando-Hernández S, Franco A, Cuevas-Nasu L, Romero-Martínez M, Hernández-Ávila M. Encuesta Nacional de Salud y Nutrición 2012. Resultados Nacionales. Cuernavaca, México: Instituto Nacional de Salud Pública (MX), 2012.

He Y, Zhang L, Zhu Z, Xiao A, Yu H, Gan X (2017) Blockade of cyclophilin D rescues dexamethasone-induced oxidative stress in gingival tissue. *PLoS ONE* 12(3): e0173270. doi:10.1371/journal.pone.0173270

Hersoug LG, Møller P, Loft S, Role of microbiota-derived lipopolysaccharide in adipose tissue inflammation, adipocyte size and pyroptosis during obesity. *Nutr Res Rev.* 2018 Jan 24:1-11. doi: 10.1017/S0954422417000269.

Hsieh C-C, Chou M-J, Wang C-H (2017) Lunasin attenuates obesity-related inflammation in RAW264.7 cells and 3T3-L1 adipocytes by inhibiting inflammatory cytokine production. *PLoS ONE* 12(2): e0171969. doi:10.1371/journal.pone.0171969

Hong Chen, Cellular inflammatory responses: Novel insights for obesity and insulin resistance, *Pharmacological Research* 53 (2006) 469–477.

Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes.* 2007; 56:901-11.

Hwang KH, Ahn JY, Kim S, Park JH, Ha TY. 2011 Abietic Acid has an anti-obesity effect in mice fed a high-fat diet. *J Med Food.* Sep;14(9):1052-6.

Ichiro Manabe, 2011, Chronic inflammation links cardiovascular, metabolic and renal diseases, *circulation journal* vol. 75.

J.A. Jeevendra Martyn, Masao Kaneki and Shingo Yasuhara, Obesity-Induced Insulin Resistance and Hyperglycemia: Etiological Factors and Molecular Mechanisms, *Anesthesiology.* 2008 July ; 109(1): 137–148.

Jaganjac M, Almuraikhy S, Al-Khelaifi F, Al-Jaber M, Bashah M, Mazloun NA, Zarkovic K, Zarkovic N, Waeg G, Kafienah W, Elrayess MA. Combined metformin and insulin treatment reverses metabolically impaired omental adipogenesis and accumulation of 4-hydroxynonenal in obese diabetic patients. *Redox Biol.* 2017 Mar 16;12:483-490. doi: 10.1016/j.redox.2017.03.012

Jaspinder Kaur, 2014, A comprehensive Review on Metabolic Syndrome, *Cardiology Research and Practice*, Volume 2014, Article ID 943162, 21 pages.

Jean-philippe Bastard, Mustapha Maachi, Claire Lagathu, Min Ji Kim, Martine Caron, Humbert Vidal, Jacqueline Capeau, Bruno Feve, Recent advances in the relationship between obesity, inflammation, and insulin resistance, *Eur. Cytokine Netw.*, Vol. 17 n° 1, 2006, pag 4-12.

Jinhan He, Chong Xub, Jiangying Kuang, Qinhui Liua, Hongfeng Jiangc, Li Mo, Bin Geng, Guoheng Xuc, 2015, Thiazolidinediones attenuate lipolysis and ameliorate dexamethasone-induced insulin resistance, *METABOLISM CLINICAL AND EXPERIMENTAL* 64 826 – 836.

J. Pedraza-Chaverrí, L.M. Reyes-Fermín, E.G. Nolasco-Amaya, et al. ROS scavenging capacity and neuroprotective effect of alpha-mangostin against 3-nitropropionic acid in cerebellar granule neurons. *Exp. Toxicol. Pathol.*, 61 (2009), pp. 491–501

Kai H, Baba M, Okuyama T. Two new megastigmanes from the leaves of *Cucumis sativus*. *Chem Pharm Bull* 2007;55:133-6.

Karen A. Harford, Clare M. Reynolds, Fiona C. McGillicuddy and Helen M. Roche, Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue, *Proceedings of the Nutrition Society* (2011), 70, 408–417.

Krauze-Baranowska M, Cisowski W. Flavonoids from some species of the genus *Cucumis*. *Biochem Syst Ecol* 2001;29:321-4.

D Kumar, S Kumar, J Singh, Narender, Rashmi, BD Vashistha, and N Singh J Young Free Radical Scavenging and Analgesic Activities of *Cucumis sativus* L. Fruit Extract. *Pharm.* 2010 Oct-Dec; 2(4): 365–368.

Kumari, K., Mathew, B.C., Augusti, K.T. 1995. Antidiabetic and hypolipidemic effects of S-methyl cysteine sulfoxide isolated from *Allium cepa* Linn. *Indian Journal of Biochemistry and Biophysics*, 32 (1), 49/54.

Laclaustra M, Corella D and Ordovas JM. Metabolic syndrome pathophysiology: the role of adipose tissue. *Nutr Metab Cardiovasc Dis.* 2007; 17: 125–39.

Liang WW, Yan J, Wu LH, Fan CL. Effect of hawthorn leaf flavonoids on lipid metabolism in hyperlipidemia rats. *Health Res* 2010; 8: 8–11

Longbiao Yao, Oana Herlea-Pana, Janet Heuser-Baker, Yitong Chen, and Jana Barlic-Dicen, Roles of the Chemokine System in Development of Obesity, Insulin Resistance, and Cardiovascular Disease *Journal of Immunology Research* Volume 2014, Article ID 181450, 11 pages.

M. Kagoshima, K. Ito, B. Cosio and I.M. Adcock. Glucocorticoid suppression of nuclear factor- κ B: a role for histone modifications. *Biochemical Society Transactions* (2003) Volume 3. PMID: 12546654 DOI: 10.1042/

Maachi M, Piéroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, Capeau J, Bastard JP. Systemic low grade inflammation is related to both circulating and adipose tissue TNF α , leptin and IL-6 levels in obese women. *Int J Obes* 2004; 28: 993.

María L. Zambrano, Deborah B. Rodríguez y Alfredo Álvarez, Kinetic Study and Surface Response Analysis on the Rehydration of Frozen-dried Carrot (*Daucus carota*), *Información Tecnológica*-Vol. 18 N°4-2007, pág.: 47-56

Maroto Borregon J.V, 2002, *Horticultura Herbacea Especial*. 5ta. Edición. Universidad politécnica de Valencia. Pag. 531-535.

Martyn, J. A, Kaneki, M. & Yasuhara, S. Obesity-Induced Insulin Resistance and Hyperglycemia: Etiological Factors and Molecular Mechanisms. *Anesthesiology* 109, 137-148 (2008).

Mata Cases Manel, 2008, *Metformina y diabetes mellitus tipo 2, Formación continuada, Atención primaria* 40(3):147-53.

Matthias Blüher. Adipokines—removing road blocks to obesity and diabetes therapy, *MOLECULAR METABOLISM* 3 (2014) 230–240.

Méndez-Martínez Marisol, 2013, “*Evaluación de la capacidad de dos extractos de diferente polaridad de Cucumis sativus para controlar las patologías asociadas a la obesidad e inducir un ambiente inmunoregulador en un modelo murino de obesidad*” [Tesis Posgrado], Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos.

Mercy Bih Achu, Elie Fokou, Clergé Tchiégang, Martin Fotso and Felicité MbiapoTchouanguep, Atherogenicity of *Cucumeropsis mannii* and *Cucumis sativus* oils from Cameroon *African Journal of Food Science*. Vol (2) pp. 021-025, March, 2008.

Monroy Ortiz C y Monroy R. 2006. Las plantas, compañeras de siempre: La experiencia en Morelos. Laboratorio de Ecología, Universidad Autónoma del Estado de Morelos. Pag. 243-245.

Mohsen Minaiyan, Behzad Zolfaghari, Amin Kamal Iranian, Effect of Hydroalcoholic and Buthanolic Extract of *Cucumis sativus* Seeds on Blood Glucose Level of Normal and Streptozotocin-Induced Diabetic Rats, *Journal of Basic Medical Sciences*. Vol. 14, No. 5, Sep-Oct 2011, 436-442.

Nabil E. Boutagy, Ryan P. McMillan, Madlyn I. Frisard, and Matthew W. Hulver Metabolic endotoxemia with obesity: is it real and is it relevant?, *Biochimie*. 2016 May; 124: 11–20. Published online 2015 Jun 29. doi: 10.1016/j.biochi.2015.06.020 PMID: PMC4695328 NIHMSID: NIHMS705683

Naik VR, Agshikar NV, Abraham GJ. 1980. Analgesic and anti-inflammatory activity in alcoholic extracts of *Cucumis trigonus* Roxburghii. A preliminary communication. *Pharmacology*;20(1):52-6.

Naito Y, Akagiri S, Uchiyama K, et al., 2005. Reduction of diabetes-induced renal oxidative stress by a cantaloupe melon extract/gliadin biopolymers, oxykine, in mice. *Biofactors*. 23(2):85-95.

Naresh Singh Gill, Shailja SOOD, Arunachalam MUTHURAMAN, Manju GARG, Raman KUMAR, Manoj BALI & Pritam. Antioxidant, Anti-inflammatory and Analgesic Potential of *Cucumis sativus* Seed Extract DEV SHARMA 3 *Lat. Am. J. Pharm.* 29 (6): 927-32 (2010)

Ning N, Han JJ. Advances in studies on active components of reducing blood lipid in traditional Chinese medicine. *Asia-Pac Tradit Med* 2013; 9: 76–79

Nuernberg K, Breier BH, Jayasinghe SN, Bergmann H, Thompson N, Nuernberg G, Dannenberger D, Schneider F, Renne U, Langhammer M, Huber K. 2011 Metabolic responses to high-fat diets rich in n-3 or n-6 long-chain polyunsaturated fatty acids in mice selected for either high body weight or leanness explain different health outcomes. *Nutr Metab (Lond)*. 11;8(1):56.

Ooi VE, Liu F., Immunomodulation and anti-cancer activity of polysaccharide-protein complexes *Curr Med Chem*. 2000 Jul;7(7):715-29.

Palomer X., Pérez A., Blanco-Vaca F., 2005, Adiponectina: un nuevo nexo entre obesidad, resistencia a la insulina y enfermedad cardiovascular, 124(10):388-95.

Peter Arner and Dominique Langin. Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance *Trends in Endocrinology and Metabolism* xx(2014)1–8.

Peter F. Surai. Silymarin as a natural antioxidant: an overview of the current evidence and perspectives. *Antioxidants*. 2015, 4(1): 204–247.

Ricardo Rodríguez-Calvo, Lucia Serrano, Norman Moullan, Xavier Palomera, Liliane Michalik, Walter Walhi y Manuel Vazquez-Carrera, PPAR β/δ reduce la expresión de citocinas inflamatorias en adipocitos mediante la inhibición de NF- κ B, *Clin Invest Arterioscl*. 2009;21(3):97-105

Rice CA, Rymal KS, Chambliss OL, Johnson FA. Chromatographic and mass spectral analysis of cucurbitacins of three *Cucumis sativus* cultivars. *J Agric Food Chem* 1981;29:194-6.

Richard Yan-Do, Patrick E. MacDonald Impaired “Glycine”-mia in Type 2 Diabetes and Potential Mechanisms Contributing to Glucose Homeostasis *Endocrinology* (2017) 158 (5): 1064-1073

R. Roman-Ramos, J.L. Flores-Saenz, F.J. Alarcon-Aguilar, Anti-hyperglycemic effect of some edible plants, *Journal of Ethnopharmacology* 48 (1995) 25-32.

Roger HF Wong and Hei Sook Sul Insulin signaling in fatty acid and fat synthesis: a transcriptional perspective *Current Opinion in Pharmacology* 2010, 10:684–691

Victoria Rotter, Ivan Nagaev and Ulf Smith, Insulin-resistant Subjects Overexpressed in Human Fat Cells from Like IL-8 and Tumor Necrosis Factor- α , Resistance in 3T3-L1 Adipocytes and Is, Interleukin-6 (IL-6) Induces Insulin, doi: 10.1074/jbc.M301977200 originally published online September 2, 2003, *J. Biol. Chem.* 2003, 278:45777-45784.

S. Artola Menéndez, M. Duelo Marcos, E. Escribano Ceruelo, Síndrome metabólico *RevPediátr Aten Primaria.* 2009;11Supl 16:s259-s277.

Sánchez-Muñoz F., García-Macedo R., Alarcón-Aguilar F. y Cruza M., 2005, Adipocinas, tejido adiposo y su relación con células del sistema inmune. *Gac Méd Méx*, 141(6).

Shafiei-Roudbari SK, Malekinejad H, Janbaz-Aciabar H, Razi M. Crosstalk between E2F1 and P53 transcription factors in doxorubicin-induced DNA damage: evidence for preventive/protective effects of silymarin. *J Pharm Pharmacol.* 2017 May 23. doi: 10.1111/jphp.12745

Schönfeld, P. & Wojtczak, L. Fatty acids as modulators of the cellular production of reactive oxygen species. *Free Radic. Biol. Med.* 45, 231–241 (2008).

Sunil K. Manna, Asok Mukhopadhyay, Nguyen T. Van and Bharat B. Aggarwal, Silymarin Suppresses TNF-Induced Activation of NF- κ B, c-Jun N-Terminal Kinase, and Apoptosis. *J Immunol* 1999, 163(12):6800-6809

Thomas H Ottens, Maarten WN Nijsten, Jan Hofland, Jan M Dieleman, Miriam Hoekstra, Diederik van Dijk and Joost MAA van der Maaten, Effect of high-dose dexamethasone on perioperative lactate levels and glucose control: a randomized controlled trial *Critical Care* (2015) 19:41

Uchida Aki, Mikhail N. Slipchenko, Ji-Xin Cheng, and Kimberly K. Buhman, 2010, Fenofibrate, A Peroxisome Proliferator-Activated Receptor α Agonist, Alters Triglyceride Metabolism In Enterocytes of Mice. *Biochim Biophys Acta.* 2011 March; 1811(3): 170–176.

Vanessa Abella, Morena Scotece, Javier Conde, Verónica López, Verónica Lazzaro, Jesús Pino, Juan J. Gómez-Reino and Oreste Gualillo. Adipokines, Metabolic Syndrome and Rheumatic Diseases, *Journal of Immunology Research*, Volume 2014, Article ID 343746, 14 pages.

Vega Robledo G. B., 2011. El adipocito y la respuesta inmune, *Revista facultad de medicina, UAEM*, Vol 50 No 1.

Veeramani C, Aristatle B, Pushpavalli G, Pugalendi KV. 2010 Antihypertensive efficacy of *Melothria maderaspatana* leaf extract on sham-operated and uninephrectomized DOCA-salt hypertensive rats. *J Basic Clin Physiol Pharmacol.*;21(1):27-41

Vidal J., Gomis R., 2008, Interrelaciones entre obesidad y aterotrombosis, *Rev Esp Cardiol Supl*, 8:3C-11C.

Vouldoukis I, Lacan D, Kamate C, et al., 2004 Antioxidant and anti-inflammatory properties of a *Cucumis melo* LC. extract rich in superoxide dismutase activity. *J Ethnopharmacol.* 94(1):67-75.

Wagner H, S Blatt (1996) *Plant Drug Analysis. A Th in Layer Chromatography Atlas.* Springer Verlag, Berlin. 384 p.

Xu C, Xu GH. Glucocorticoids, adipose metabolism and insulin resistance. *Sheng Li Ke Xue Jin Zhan*. 2009, 40(1):19-23

Yazmín Reyes López, Israel Pérez-Torres, Alejandra Zúñiga-Muñoz, Verónica Guarner Lans, Eulises Díaz-Díaz, Elizabeth Soria Castro and Rodrigo Velázquez Espeje. Effect of Glycine on Adipocyte Hypertrophy in a Metabolic Syndrome Rat Model *Current Drug Delivery*, 2016, 13, 158-169

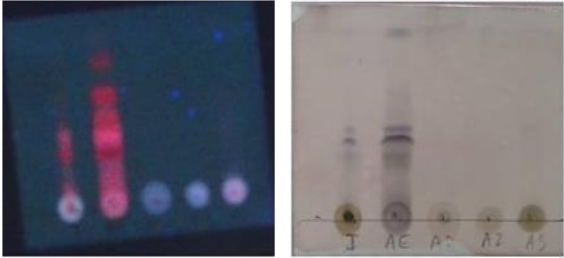
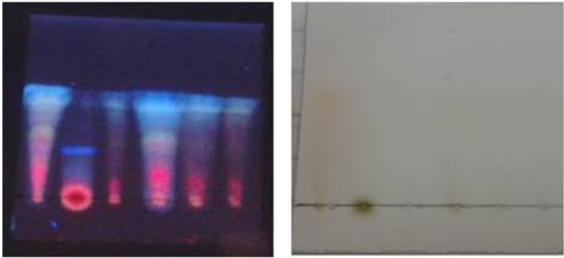
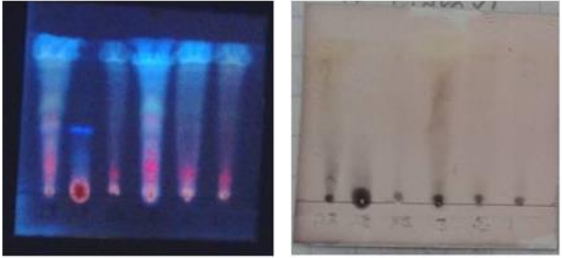
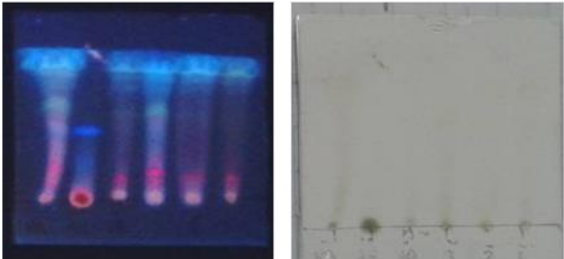
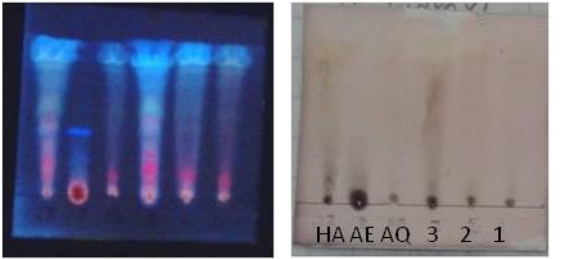
Ying Lin, Hyangkyu Lee, Anders H. Berg, Michael P. Lisanti, Lawrence Shapiro, and Philipp E. Schereri. The Lipopolysaccharide-activated Toll-like Receptor (TLR)-4 Induces Synthesis of the Closely Related Receptor TLR-2 in Adipocytes. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* Vol. 275, No. 32, Issue of August 11, pp. 24255–24263, 2000

Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 2000; 148: 209.

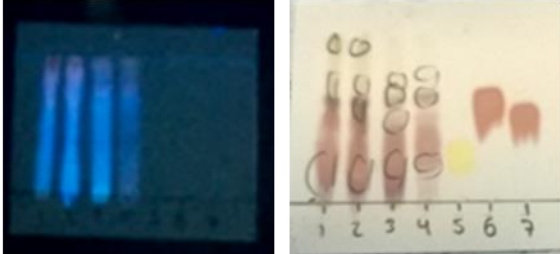
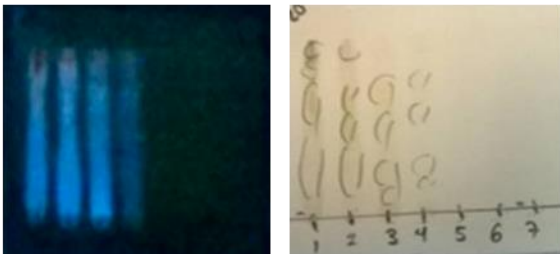
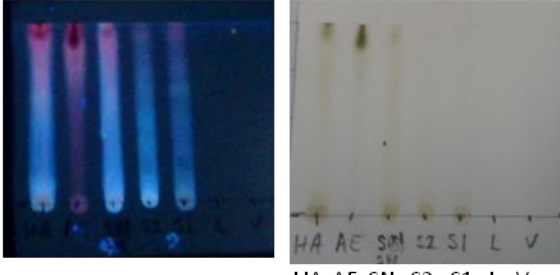

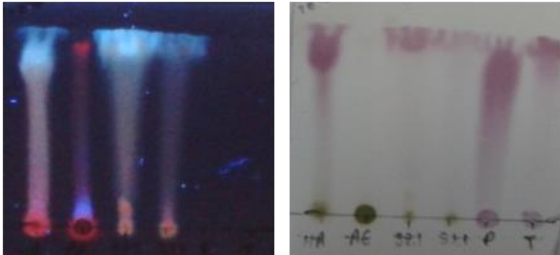
Zhong XL, Ding JL, Luo DS. Progress on blood lipidregulating active ingredients from traditional Chinese medicine. *Food & Drug* 2010; 11: 449–451

Zuhair HA, Abd El-Fattah AA, El-Sayed MI. . 2000. Pumpkin-seed oil modulates the effect of felodipine and captopril in spontaneously hypertensive rats. *Pharmacol Res* 41(5):555-63

Anexo 1. Cromatografías en Capa Fina (CCF) del extracto acuoso y fracciones de *Cucumis sativus*

Condiciones	Placas	Muestras
<p>A) Fase Normal Sistema: CH₃Cl : MeOH Proporción 9 : 1 Revelador: 4 Hidroxibenzaldehído</p>		<p>1=Hidroalcolólico AE= Acetato de Etilo 1=Sólido 1 2=Sólido 2 3=Sobrenadante</p>
<p>B) Fase Reversa Sistema: H₂O : CH₃CN Proporción 7: 3 Revelador: Flavonoides</p>		<p>1=Hidroalcolólico 2=Acetato de etilo 3=Acuoso 4=Sobrenadante 2 5= Sólido 2 6= Sólido 1</p>
<p>C) Fase Reversa Sistema: H₂O : CH₃CN Proporción 7: 3 Revelador: 4 Hidroxibenzaldehído</p>		<p>1=Hidroalcolólico 2=Acetato de etilo 3=Acuoso 4=Sobrenadante 2 5= Sólido 2 6= Sólido 1</p>
<p>D) Fase Reversa Sistema: H₂O : CH₃CN Proporción 7: 3 Revelador: Naftol</p>		<p>1=Hidroalcolólico 2=Acetato de etilo 3=Acuoso 4=Sobrenadante 2 5= Sólido 2 6= Sólido 1</p>
<p>E) Fase Reversa Sistema: H₂O : CH₃CN Proporción 7: 3 Revelador: 4 Hidroxibenzaldehído</p>		<p>HA=Hidroalcolólico AE= Acetato de Etilo AQ=Acuoso 3=Sobrenadante 2=Sólido 1 1=Sólido 2</p>

Continuación del Anexo 1. Cromatografías en Capa Fina (CCF) del extracto acuoso y fracciones de *Cucumis sativus*

Condiciones	Placas	Muestras
<p>G) Fase Normal Sistema: nBuOH:acetona:Hac:H₂O Proporción 35:35 : 1: 2 Revelador: Ninhidrina</p>		<p>1=Hidroalcolólico 2=Sobrenadantes 3=Sólido 2.2 4=Sólido 1 5= Prolina 6= Leucina 7= Valina</p>
<p>H) Fase Normal Sistema: nBuOH:acetona:Hac:H₂O Proporción 35:35:1:2 Revelador: Flavonoides</p>		<p>1=Hidroalcolólico 2=Sobrenadantes 3=Sólido 2.2 4=Sólido 1 5= Prolina 6= Leucina 7= Valina</p>
<p>I) Fase Normal Sistema: nBuOH:acetona:Hac:H₂O Proporción 7: 7 : 1: 2 Revelador: Ninhidrina</p>		<p>HA=Hidroalcolólico AE= Acetato de Etilo SN=Sobrenadantes S2= Sólido 2.2 S1=Sólido 1 L=Lignano V=Valina</p>
<p>J) Fase Normal Sistema: nBuOH:acetona:Hac:H₂O Proporción 7: 7 : 1: 2 Revelador: Ninhidrina</p>		<p>HA=hidroalcohólico A= 6 dimetilamino purina (dimetil adenina) G= guanosina 5'-O-3' Thiotrifosfato L= lignano</p>
<p>K) Fase normal Agua:acetonitrilo Proporción 8:2 Ninhidrina</p>		<p>HA AE Solido amarillo Solido 1 Fenilalanina Treonina</p>