UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE CIENCIAS QUÍMICAS, INGENIERÍA Y MEDICINA

PROGRAMAS MULTIDISCIPLINARIOS DE POSGRADO EN CIENCIAS AMBIENTALES

TESIS QUE PARA OBTENER EL GRADO DE

DOCTORADO EN CIENCIAS AMBIENTALES

EVALUACIÓN TÓXICA (*in vivo*) Y BIOLÓGICA (*in vitro*) DEL EXTRACTO ACUOSO LIOFILIZADO DE *Calea urticifolia* (MILL) DC.

PRESENTA:

M. en C. María Lucina Torres Rodríguez

DIRECTOR DE TESIS:

Dra. Erika García Chávez

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Dra. Celia Aradillas García

Dra. Ana Cristina Cubillas Tejeda

Diciembre 2015

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A Dios, por ser el motor y fuerza que impulsa mi vida en todo momento.

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RESUMEN

La inflamación de bajo grado del tejido adiposo (IBTA), se relaciona ampliamente con una variedad de trastornos metabólicos y enfermedades crónicas degenerativas tales como obesidad, resistencia a la insulina, diabetes tipo 2, enfermedad cardiovascular y cáncer. El té preparado con las hojas de *Calea urticifolia* se usa tradicionalmente como remedio para tratar ulceras gástricas, diabetes y procesos inflamatorios por el grupo étnico Xi'iuy de la región de La Palma en el municipio de Rayón-Tamasopo de San Luis Potosí, México. Estudios previos corroboran sus efectos etno-farmacológicos; en un modelo *in vivo* de inflamación aguda mediada por carragenina se evaluó el extracto acuoso y el extracto etanólico. En otro estudio se valoró el efecto anti-inflamatorio del extracto etanólico en un modelo *in vivo* de IBTA. Los resultados indican que la planta posee un potencial anti-inflamatorio aunado al de presentar efectos como hipoglucemiante y anti-lipemiante en el IBTA. A pesar de la valía de este conocimiento científico sobre su efecto terapéutico, no hay evidencias que fundamenten la seguridad del extracto acuoso liofilizado de *C. urticifolia* (CuAqE) para que se permita un estudio de intervención clínica y sus efectos terapéuticos. Por lo tanto, esta investigación tuvo como objetivo contribuir al estudio de la toxicidad aguda y sub-crónica del CuAqE *in vivo*, así como la caracterización química del extracto y su participación en los mecanismos asociados a su efecto farmacológico como anti-inflamatorio y anti-oxidante *in vitro*. Con este fin se utilizaron ratas Wistar en los ensayos *in vivo*. La evaluación de la toxicidad aguda del CuAqE y del extracto etanólico se realizó por administración oral única de dosis fijas de 50, 100, 300, 1000, 2000 y 5000 mg/kg de peso. Los efectos adversos y la mortalidad se registraron durante 14 días, obteniéndose una dosis letal $(DL)_{50}$ > 1000 mg/kg con el extracto etanólico y una DL_{50} > 5000 mg/kg con el extracto acuoso de *C. urticifolia*. La toxicidad sub-crónica se evaluó únicamente con el CuAqE a dosis de 0.5, 50

y 500 mg/kg/28 días vía oral. Durante el estudio se registraron diariamente el consumo de alimento, de agua y el peso corporal. Al final del experimento se registró el peso de órganos y se cuantificaron parámetros bioquímicos y hematológicos. Los resultados mostraron ausencia de mortalidad y de signos clínicos tóxicos posteriores a la exposición. Sin embargo, en ratas hembra, la ganancia del peso corporal fue significativamente menor que en el grupo control ($p < 0.05$) con la dosis de 50 mg/kg de CuAqE los días 21 y 28 (28 ± 25% y 44 ± 25%, respectivamente) y en la dosis de 500 mg/kg los días 7, 14, 21 y 28 del experimento (95 ± 66%, 64 ± 45%, 59 ± 31% y 64 ± 19%, respectivamente).

Los compuestos fenólicos del CuAqE se caracterizaron por Cromatografía Líquida con Ionización en Electro Spray Acoplada a Masas (LC-ESI-MS) y se encontró una mezcla de los derivados del ácido cafeoil-quínico, así como flavonoides glicósidos en el CuAqE. Se evaluaron los efectos terapéuticos *in vitro* en macrófagos RAW 264.7 estimulados con 1 µg/mL de lipopolisacarido (LPS) y tratados con 10, 25, 50, 75 y 100 µg/mL de CuAqE y se cuantificaron parámetros pro-inflamatorios y especies reactivas de oxígeno (ROS). Los resultados sugieren que el efecto anti-inflamatorio es mediado por la supresión de la vía del óxido nítrico (NO)/óxido nítrico sintetasa inducible (iNOS) a través de inhibición de la tras locación de las subunidades p65 y p50 del factor nuclear kappa B (NF-κB). Con respecto a su efecto anti-oxidante, la producción de ROS se inhibió significativamente ($p < 0.05$) en forma dosis-dependiente. Además, la expresión de marcadores de inflamación disminuyó con 50 µg/kg de CuAqE en un 34.5 a 88.3%.

Finalmente, los resultados de la evaluación tóxica clasifican al CuAqE con toxicidad baja. Por lo tanto, sugieren que la dosis del té de *C. urticifolia* (equivalente a 0.55 mg CuAqE/kg de peso corporal) que consume la comunidad Xi'iuy es segura, debido a que se encuentra 9091veces por debajo de la DL_{50}. Se sugiere que los compuestos fenólicos presentes en el CuAqE pueden ser los

responsables de inhibir la inflamación inducida por LPS en macrófagos RAW 264.7 a través de la vía NO/iNOS y la supresión de la vía de señalización del NF-κB así como por la inhibición de la producción de ROS.

ABSTRACT

Low-grade inflammation of the adipose tissue (IBTA) has been widely related with metabolic disorders and chronic degenerative diseases such as obesity, insulin resistance, type 2 diabetes, cardiovascular disease and cancer. The tea prepared with *Calea urticifolia* leaves is traditionally used as a remedy to treat gastric ulcers, diabetes and inflammatory processes by Xi'iuy ethnic group of La Palma region in the municipality of Rayon-Tamasopo of San Luis Potosi, Mexico. Previous studies have corroborated its traditional uses; on *in vivo* model of acute inflammation mediated by carrageenan aqueous extract and the ethanol extract was evaluated. On the other hand, anti-inflammatory effect of ethanolic extract was evaluated in an IBTA chronic model. These results position to vegetable species as a potential anti-inflammatory plant, also presenting effects as hypoglycemic and anti-lipid effects of IBTA condition. Despite the value of this scientific knowledge about its therapeutic effect, there is no evidence to support the safety of lyophilized aqueous extract of *C. urticifolia* (CuAqE) and justifying its therapeutic effects in clinical intervention study. Therefore, the objective of this research was to evaluate acute and sub-chronic toxicity *in vivo* of CuAqE, as well as to characterize fenolic composition and to evaluate *in vitro* the mechanisms associated with its anti-inflammatory and anti-oxidant effect. Wistar rats were used in *in vivo* assays. Acute toxicity test of CuAqE and ethanolic extract were performed by administration of fixed single doses at 50, 100, 300, 1000, 2000 and 5000 mg/kg given by gavage. General behavior, adverse effects and mortality were recorded during the 14 days. Results showed a LD_{50} > 1000 mg/kg for the ethanol extract and > 5000 mg / kg for the aqueous extract of *C. urticifolia*. Sub-chronic toxicity was evaluated only with CuAqE at doses of 0.5, 50 and 500 mg/kg/28 days by administration gavage. Food and water consumption and body weight were daily registered during experiment. At the end of study, hematological and

biochemical parameters were determined and organs weight was registered. Results showed no mortality and no clinical signs toxic. However, in female rats body weight gain was significantly lower than the control group (p <0.05) by 50 mg / kg at 21 and 28 (28 ± 25% and 44 ± 25% respectively) and by 500 mg / kg at 7, 14, 21 and 28 day (95 ± 66%, 64 ± 45%, 59 ± 31% and 64 ± 19% respectively).

Phenolics compounds of CuAqE were characterized by Liquid Chromatography-Electro Spray Ionization-Mass Spectrometry (LC-ESI-MS) and a mix of caffeoyl-quinic acid derivatives and flavonoid glycosides were found. Therapeutic effects of CuAqE were evaluated *in vitro* model on RAW 264.7 macrophages treated with 10, 25, 50, 75 and 100 µg/mL of extract and stimulated with 1 µg/mL lipopolysaccharide (LPS). Pro-inflammatory parameters and reactive oxygen species (ROS) were measured. Results suggest that anti-inflammatory effect is mediated by suppression of the nitric oxide (NO) / inducible nitric oxide synthase (iNOS) through inhibition of trans-location of p65 and p50 NF-κB sub-units. ROS production was significantly (p < 0.05) inhibited in a dose-dependently manner. In addition the expression of markers of inflammation was suppressed by 50 mg/kg of CuAqE (34.5 to 88.3 %).

Finally, results of toxic evaluation classify to CuAqE with low toxicity. Therefore, this research suggest that dose of *C. urticifolia* tea (equal to 0.55 CuAqE mg/kg body weight) used by Xi'iuy community is safe, because it represents 9091 times below LD_{50} tested in toxicity study. Results suggest that phenolic compounds in the CuAqE may be responsible to inhibit LPS-induced inflammation by iNO/NO pathway through suppressing NF-kB signaling pathway and inhibition of ROS.

I. INTRODUCCIÓN

La obesidad es un problema grave de salud pública a nivel mundial ya que representa un riesgo elevado para desarrollar enfermedad cardiovascular, diabetes tipo 2 (DT2) y cáncer (Abel *et al.,* 2008; Balistreri *et al.,* 2010; De Pergola y Silvestris, 2013; Anderson *et al.,* 2014). La obesidad es una patología multifactorial que resulta de un desbalance energético entre las calorías consumidas y las calorías gastadas y se define como una acumulación anormal y excesiva de grasa que deteriora la salud (WHO, 2015). Además, la obesidad forma parte del síndrome metabólico y se considera un factor de riesgo para desarrollar resistencia a la insulina (Faloia, 2012; Xu *et al.,* 2015) y se asocia con un estado de inflamación crónica de bajo grado, que se caracteriza por la infiltración de macrófagos activados en el tejido adiposo disfuncional (Samaan *et al.,* 2011). Este proceso sistémico implica la activación de vías de señalización intracelular que desencadenan la secreción de varios mediadores pro-inflamatorios como el óxido nítrico, la prostaglandina E_2, citocinas como el factor de necrosis tumoral (TNF) α, las interleucinas (IL) -1β y -6 y adipocinas como leptina y resistina, entre otros. Más aún, el factor de transcripción nuclear-κ B (NF-κB) juega un papel primordial en el desarrollo y persistencia de este proceso (Tilg y Moschen, 2008).

En los últimos años, la obesidad se considera una pandemia ya que es un problema grave de salud pública en países de ingresos económicos elevados y también en aquellos donde los ingresos son bajos (WHO, 2015). Actualmente, México se considera uno de los países con los índices de sobrepeso y obesidad más altos, ya que se presenta tanto en adultos como niños de comunidades urbanas e indígenas (ENSANUT, 2012). Por lo que es importante resaltar que las comunidades indígenas no se encuentran exentas de desarrollar las patologías que se asocian con la obesidad como enfermedad cardiovascular, DT2 y cáncer. Más aún, este sector de la población

resulta ser uno de los más vulnerables y recurre al uso de la medicina tradicional como su principal, y en ocasiones única, fuente para resolver sus problemas de salud. Es por eso que resulta esencial fomentar el uso racional y adecuado de la medicina tradicional, así como identificar, valorar y documentar terapias seguras y eficaces para proteger a quienes la consumen (WHO, 2014).

La medicina tradicional se utiliza desde tiempos ancestrales y representó la fuente terapéutica principal de nuestros antepasados. Actualmente, se reconoce como el conjunto de conocimientos, capacidades y prácticas basadas en teorías, creencias y experiencias de los diferentes pueblos indígenas del mundo. Sin embargo, a pesar de que la medicina moderna ha logrado erradicar enfermedades severas del presente y del pasado, la práctica de la medicina tradicional sigue vigente en las comunidades indígenas y ha resurgido en países desarrollados así como también en aquellos en vías de desarrollo. Además, la Organización Mundial de la Salud (OMS) estima que un 80% de la población mundial, principalmente de países pobres, utiliza la medicina tradicional como su principal fuente de cuidados terapéuticos.

Calea urticifolia es una planta medicinal usada por el grupo étnico Xi'iuy de San Luis Potosí, México. Se encuentra ampliamente distribuida desde México hasta Panamá en climas cálido y semi-cálido, se asocia con ecosistemas de selva baja caducifolia, bosque tropical y de encino (Biblioteca Digital de la Medicina Tradicional Mexicana, 2009). El té preparado con las hojas de *C. urticifolia* se usa tradicionalmente como remedio para tratar diabetes, ulceras gástricas y procesos inflamatorios (Guzmán, 2010). Estudios previos han determinado que *C. uriticifolia* contiene lactonas sesquiterpenas con esqueleto de germacrano (Yamada *et al.*, 2004). Estos compuestos químicos tienen actividad biológica importante tal como la inhibición de la biosíntesis de melanina (Ohguchi *et al*, 2009), la inducción de apoptosis en la línea celular HL60

(Nakagawa *et al*, 2005), efecto anti-oxidante (Umemura *et al*, 2008) y supresión de la diferenciación de adipocitos 3T3 L1 (Matsuura *et al*, 2005). Además, estudios recientes de nuestro grupo de trabajo mostraron que el extracto etanólico de las hojas de *C. urticifolia*, resultó ser un agente anti-inflamatorio eficaz, capaz de disminuir la secreción de citocinas pro-inflamatorias (TNF-α, IL-1β e IL-6) en un modelo *in vivo* de inflamación crónica de bajo grado (Guzmán, 2010; Ortiz, 2011). Sin embargo, a pesar de la evidencia científica de los efectos farmacológicos de esta especie, los estudios sobre la evaluación tóxica, composición química y actividad biológica del extracto acuoso de *C. urticifolia* son limitados. Por lo tanto, el objetivo de esta investigación fue valorar la capacidad tóxica del extracto de *C. urticifolia in vivo*, caracterizar la composición química de los compuestos fenólicos y evaluar la actividad biológica *in vitro* del efecto anti-inflamatorio y anti-oxidante del extracto acuoso liofilizado de las hojas de *C. urticifolia*.

Finalmente, los hallazgos de esta investigación apoyan la validación científica del efecto anti-inflamatorio y anti-oxidante que se le atribuye de manera tradicional a la especie. Además, la evaluación toxicológica contribuye a considerar al extracto acuoso liofilizado de *C. urticifolia* como una sustancia de baja toxicidad que puede considerarse segura para su consumo. Por tanto, estos resultados contribuyen a la investigación pre-clínica de la especie, que servirá de antecedente para realizar estudios clínicos posteriores.

II. ARTÍCULOS

Capítulo I. Evaluación de la toxicidad aguda *in vivo* del extracto etanólico y acuoso de *Calea urticifolia*.

Capítulo II. Sub-chronic oral toxicity of *Calea urticifolia* lyophilized aqueous extract in Wistar rats.

Capítulo III. Anti-inflammatory and anti-oxidant effect of *Calea urticifolia* lyophilized aqueous extract on lipopolysaccharide-stimulated RAW 264.7 macrophages through suppressing nuclear factor NF-κB signaling pathway.

Capítulo I.

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Evaluación de la toxicidad aguda *in vivo* del extracto etanólico y acuoso de *Calea urticifolia*

Evaluation *in vivo of* acute toxicity of ethanolic and aqueous extract of *Calea urticifolia*

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Resumen

Calea urticifolia es reconocida por su eficacia en el tratamiento de enfermedades relacionadas con procesos anti-inflamatorios. Sin embargo, a pesar de la corroboración científica de su efecto etno-farmacológico, no existe evidencia de estudios toxicológicos sobre esta especie con amplio uso en la medicina tradicional en la comunidad Xi'iuy del estado de San Luis Potosí, México. Por

lo anterior, en este trabajo estudiamos la toxicidad aguda de los extractos etanólico y acuoso de las hojas de *C. urticifolia*. Se utilizaron ratas Wistar de ambos sexos para determinar la dosis letal $(DL)_{50}$ utilizando el Método Alternativo de Clases por vía oral. Las dosis fijas evaluadas fueron 50, 100, 300, 1000, 2000 y 5000 mg/kg de peso. Se evaluó el cambio en la ganancia de peso corporal y parámetros de funcionalidad renal y hepática. Los resultados mostraron que el extracto etanólico tenía una DL_{50} > 1000 mg/kg y que el extracto acuoso tenía una DL_{50} > 5000 mg/kg y ninguno produjo signos o síntomas de toxicidad aguda y no produjo alteraciones en la ganancia de peso corporal. La concentración sérica de urea, creatinina y transaminasas (TGP y TGO) no mostró cambios significativos con respecto al grupo control, por lo que el extracto se puede clasificar como un producto de categoría toxicológica 4 en su forma etanólica y categoría 5 en su forma acuosa, de acuerdo al Sistema Mundialmente Armonizado de Clasificación de Sustancias (GHS; Globally Harmonized Classification System).

Palabras clave: *Calea urticifolia*, medicina tradicional, toxicidad aguda.

Abstract

Calea urticifolia is recognized for its efficacy in the treatment of diseases associated with inflammatory processes; however, despite of the scientific corroboration of its traditional effect, there is no evidence of toxicological studies of the species widely used in traditional medicine in native community Xi'iuy of San Luis Potosi, Mexico. Therefore, in this work we studied acute toxicity of ethanolic and aqueous extracts of the leaves of *C. urticifolia*. Both genders of Wistar rats were used to determine the LD_{50} using the Alternative Oral Acute Toxic Class Method. The fixed doses evaluated in this study were 50, 100, 300, 1000, 2000, and 5000 mg/kg. We evaluated changes in body weight gain and parameters of renal and liver functionality. The

results showed a LD_{50} > 1000 mg/kg for the ethanolic extract and > 5000 mg/kg for the aqueous extract and any extract produced signs or symptoms of acute toxicity. In addition, did not alter body weight gain. The serum concentration of urea, creatinine and serum transaminase levels showed no significant changes compared with the control group. Based in these results, extract of *C. urticifolia* can be classified as a substance category 4 in its ethanolic form and as category 5 in the aqueous form, according to GHS Globally Harmonized Classification System (GHS).

Key words: acute toxicity, *Calea urticifolia,* traditional medicine.

Calea urticifolia es un arbusto de la familia Asteraceae que mide de 1 a 3 m de altura, se distribuye ampliamente desde México hasta Panamá en climas cálidos y semicálidos, se asocia con ecosistemas de selva baja caducifolia, bosque tropical y de encino. Se le conoce comúnmente como hierba del negro, negrito, jaral de castilla, chilchaca (Biblioteca digital de la Medicina Tradicional Mexicana, 2009) y como juanislama en el Salvador (Matsuura *et al.*, 2005).

Estudios previos sobre la composición química de *C. urticifolia* muestran que posee lactonas sesquiterpénicas (Yamada *et al*., 2004). En la literatura se reporta que estos compuestos químicos, presentes en extractos orgánicos de las hojas de *C. urticifolia*, generan apoptosis en la línea celular tumoral HL60, activan la vía Nrf2/ARE que previene el daño oxidativo en células de feocromocitoma PC12 de rata, inhiben la síntesis de melanina en células de melanoma de ratón B16 e inhiben la diferenciación de los pre-adipocitos en la línea celular 3T3-L1, siendo así un inhibidor de la adipogenesis (Matsuura *et al.*, 2005; Nakagawa *et al*., 2005; Umemura *et al.*, 2008; Ohguchi *et al*., 2009). Sin embargo, los estudios relacionados con la evaluación farmacológica del uso tradicional son limitados.

En San Luis Potosí, México; el grupo étnico Xi'iuy o pame usa tradicionalmente la decocción de las hojas de *C. urticifolia* tomada en ayunas (0.13 g de hojas secas en 256 ml de agua, correspondiente a una dosis aproximada de 0.276 mg de extracto acuoso seco/kg de peso corporal/día) como remedio terapéutico para tratar problemas gastrointestinales, diabetes e inflamación (Guzmán-Guzmán, 2010). Su uso tradicional como anti-inflamatorio se corroboró al evaluar el extracto acuoso y etanólico en un modelo *in vivo* de inflamación aguda y el extracto etanólico en un modelo *in vivo* de inflamación crónica de bajo grado del tejido adiposo, donde se observó la capacidad de inhibición sobre la secreción de citocinas pro-inflamatorias como el factor de necrosis tumoral (TNF)-α, la interleucina (IL)-6 e IL-1β, tal efecto clasifica a la especie como un agente bio-activo eficaz que inhibe el proceso inflamatorio (Guzmán-Guzmán, 2010; Ortiz-Segura, 2011; Torres-Rodríguez *et al.*, 2014).

La importancia del estudio de plantas medicinales sobre el proceso de inflamación de bajo grado que se presenta en la obesidad, ha generado un amplio interés de investigación debido a que las enfermedades relacionadas con este proceso como la diabetes mellitus tipo 2, la enfermedad cardiovascular y en algunos tipos de cáncer, representan un problema de salud pública de importancia en México y en el mundo (Oviedo *et al*., 2007; Balistreri *et al*., 2010; OMS, 2014).

En la actualidad ha incrementado el interés por el uso de las plantas medicinales y la Organización Mundial de la Salud (OMS) estima que más del 80% de la población mundial, principalmente de países en vías de desarrollo, utiliza la medicina tradicional como parte de su atención primaria de salud y que gran parte de los tratamientos tradicionales comprenden el uso de extractos de plantas o sus principios activos (OMS, 2008), por lo que es importante establecer la seguridad del uso de estas plantas medicinales.

Por tanto, y con la finalidad de contribuir a la investigación pre-clínica de *Calea urticifolia* y de validar científicamente la inocuidad de las plantas medicinales, se evaluó el efecto tóxico de los extractos etanólico y acuoso de las hojas de la especie *Calea urticifolia* mediante la determinación de la DL_{50} por el método alternativo de clases de toxicidad oral aguda.

Materiales y métodos

Material vegetal. Se recolectaron las hojas de plantas adultas en fase vegetativa de *Calea urticifolia* en la comunidad indígena Xi'iuy del Potrero del Carnero del municipio de Rayón, San Luis Potosí, México en agosto de 2012. El material vegetal se colocó en papel absorbente a temperatura ambiente y al abrigo de la luz para secarse.

Preparación de los extractos de Calea urticifolia. El extracto etanólico se preparó por maceración de las hojas trituradas y secas con etanol absoluto en una proporción 1:4 (peso/volumen) durante diez días. Se filtró y se evaporó parcialmente el alcohol a presión reducida (600 mmHg) y temperatura de 40˚C en un rotavapor (Büchi RE111). Finalmente, el extracto se colocó en cápsulas de porcelana (estandarizadas en peso constante) a temperatura ambiente y al abrigo de la luz para eliminar totalmente el solvente restante.

La extracción acuosa se llevó a cabo por decocción de100 g de hojas trituradas y secas en un litro de agua destilada a ebullición durante 5 min. Una vez frío el extracto acuoso, se filtró y se liofilizó en un Freeze dryer (Modelo TFD5505, Ilshin®). El rendimiento de la extracción etanólica y acuosa se determinó por peso de extracto seco/100 g de planta seca.

Modelo animal. Se utilizaron ratas macho y hembra de la cepa Wistar de 8 a12 semanas de edad, con peso corporal de 180 a 240 g. Se colocaron en jaulas de acrílico individuales a una temperatura de 22 ± 3˚C y bajo ciclos de luz/oscuridad de 12 h. Se les proporcionó dieta estándar (Formulab Chow 5001) y agua *ad libitum* durante el tiempo de adaptación (5 días) y experimentación (14 días). El Bioterio de la Facultad de Medicina de la Universidad Autónoma de San Luis Potosí proporcionó los animales y éstos se manejaron de acuerdo con la Norma Oficial Mexicana NOM-062-ZOO-1999, según las especificaciones técnicas de producción, cuidado y uso adecuado de animales de laboratorio (NOM 062-ZOO, 1999).

Toxicidad aguda por el Método de Clases. La DL_{50} se determinó utilizando el método alternativo de clases de toxicidad aguda por vía oral de acuerdo con la guía 423 de la Organización para la Cooperación y Desarrollo Económicos (OCDE) (OECD, 2000). Este método cumple con el principio de las tres R (reducción, refinamiento y reemplazo) y dispone la evaluación toxicológica en grupos reducidos de animales de experimentación a dosis fijas durante 14 días (Seidle *et al*, 2010).

Para la evaluación de la DL_{50} se utilizaron 36 ratas hembra y 36 macho que se distribuyeron aleatoriamente en grupos de tres en función de los tratamientos. Con ayuno previo de 12 h, se administraron los extractos etanólico o acuoso de *C. urticifolia* en la dosis correspondiente por vía oral a través de una cánula intra-gástrica (16G). Posterior a la administración aguda de los tratamientos, y con la finalidad de favorecer la absorción de los extractos, el alimento y el agua *ad libitum* se administraron 4 h después, de acuerdo con lo que se establece en la técnica experimental de la guía 423 de la OCDE.

La observación de los animales se inició inmediatamente después de administrar los tratamientos y se dirigió principalmente a la ocurrencia de muertes para determinar la DL_{50}, así

como a la aparición de signos tóxicos de bajo grado relacionados con el estado general de salud y comportamiento de las ratas (lagrimeo, apnea, disnea, salivación, temblor, somnolencia, letargo, ruido nasal, piloerección, epistaxis y convulsiones). El primer día de experimentación se registraron las observaciones a las 0.5, 4, 8, 12, 16, 20 y 24 h, y posteriormente cada 24 h hasta completar los 14 días del ensayo.

Las dosis aplicadas del extracto acuoso liofilizado fueron 50, 100, 300, 2000 y 5000 mg/kg (disueltas en agua destilada) y del extracto etanólico fueron de 50, 100 mg/kg (disueltas en agua destilada), 300 y 1000 mg/kg (disueltas en aceite de maíz). El grupo control se trató con agua destilada o aceite de maíz, respectivamente. De manera adicional se registró el peso corporal de los animales, así como el consumo de alimento y agua en los días 1, 7 y 14.

Parámetros bioquímicos. Con la finalidad de determinar cambios bioquímicos en la funcionalidad renal y hepática se extrajo una muestra sanguínea a través de la vena caudal por técnica de goteo antes de la administración de los tratamientos (basal) a los animales para determinar el intervalo de referencia por género y otra al final del experimento (pos-tratamiento) para evaluar los cambios relacionados con la exposición aguda a los extractos.

Las muestras biológicas se centrifugaron a 3000 rpm durante 5 min a temperatura ambiente, se separó el suero, se etiquetó y se almaceno a -20°C hasta su posterior análisis.

Se cuantificó la concentración sérica de creatinina por el método colorimétrico-cinético de Jaffé a 492 nm y de urea por el método de ureasa GLDH cinético a 340 nm en un instrumento RA-50 de Bayer para evaluar la funcionalidad renal. La funcionalidad hepática se determinó mediante la concentración de las enzimas transaminasas glutámico pirúvica (TGP) y glutámico oxalacética (TGO) por el método de NADH cinético UV a 340 nm en un instrumento RA-50 de Bayer.

Análisis estadístico. Los parámetros cuantitativos se expresaron como la media ± desviación estándar (DE) y se utilizó el paquete estadístico Stata versión 13.0 para realizar el análisis estadístico de los datos. Se presentó estadística descriptiva y la significancia se evaluó mediante un análisis de varianza (ANOVA), seguido de una comparación múltiple de medías de Bonferroni para determinar la diferencia entre tratamientos. Se consideró un valor de $p \leq 0.05$ como estadísticamente diferente. Se utilizó el análisis de mediciones repetidas para las variables con medidas en el tiempo.

Resultados

Rendimiento de la extracción. Por cada 100 g de hojas secas de *C. urticifolia* se obtuvieron 14.88 g de extracto acuoso seco y 2.44 g de extracto etanólico seco.

Toxicidad aguda por el Método de Clases. La administración oral del extracto etanólico (50, 100 300 y 1000 mg/kg) y del extracto acuoso (50, 100, 300, 2000 y 5000 mg/kg) en ratas hembra y macho de la cepa Wistar no causaron mortalidad en los 14 días del ensayo. Por lo que la DL_{50} en ratas Wistar y en ambos géneros es >1000 mg/kg para el extracto etanólico y > 5000 mg/kg para el extracto acuoso.

Se observaron signos de toxicidad de menor grado caracterizados por letargo, somnolencia y piloerección moderada durante las primeras 24 h de exposición en un animal tratado con 1000 mg/kg del extracto etanólico en ambos géneros y en dos ratas hembra de la dosis de 5000 mg/kg del extracto acuoso. Tales signos revirtieron al día siguiente de la exposición. En general, los grupos tratados no mostraron anormalidades en su comportamiento, ni en el estado físico o afecciones gastrointestinales caracterizados por diarrea o vómito.

Ganancia de peso corporal, consumo de alimento y agua. El peso corporal es un indicador importante de la toxicidad aguda de una sustancia, esta variable depende directamente del consumo de alimento y agua en la rata. Los resultados se presentan como la ganancia de peso corporal en porcentaje durante los días 1, 7 y 14 de observación con respecto al peso inicial. Las ratas hembra tratadas con el extracto etanólico de *C. urticifolia* (50, 100, 300 y 1000 mg/kg) no mostraron modificaciones en la ganancia de peso corporal en comparación con el grupo control (Figura 1A). Las ratas macho tuvieron una disminución significativa de la ganancia de peso corporal con respecto al control con las dosis de 100 y 1000 mg/kg en el día 7 (Figura 1B).

El extracto acuoso de *C. urticifolia* no mostró efectos sobre la ganancia de peso corporal en los grupos de ratas hembra con respecto al grupo control (Figura 2A) y en las ratas macho, la administración de las dosis de 100 y 300 mg/kg redujo ganancia de peso corporal con respecto al grupo control en los días 1 y 7 pues al día 14 se observó una recuperación del peso corporal en ambos grupos (Figura 2B).

Los grupos de ratas hembra tratadas no mostraron efectos significativos sobre el consumo de agua y alimento durante todo el tiempo de experimentación (Cuadro 1). En el caso de las ratas macho, únicamente la dosis de 50 mg/kg de extracto acuoso produjo una disminución significativa en el consumo de agua con respecto al grupo control el día 14 del experimento (Cuadro 2).

Parámetros bioquímicos. Se evaluó la funcionalidad renal a través de la cuantificación de urea y creatinina, así como la funcionalidad hepática mediante la cuantificación de enzimas transaminasas glutámico pirúvica (TGP) y glutámico oxalacética (TGO) y se comparó con los valores basales establecidos que se encuentran en el Cuadro 3. Los resultados sobre la funcionalidad renal y hepática en el grupo de ratas expuestas al extracto etanólico en ambos

géneros no presentaron cambios significativos al compararse con su respectivo control (agua *vs.* 50 y 100 mg/kg, y aceite de maíz *vs.* 300 y 1000 mg/kg). Mientras que en los animales tratados con el extracto acuoso (Cuadro 5), únicamente el grupo de ratas hembra expuestas a la dosis de 2,000 mg/kg presentó un incremento significativo en la concentración de urea de 64 ± 2 mg/dL en comparación con el grupo control (agua) 45,75 ± 9.14 mg/dL (p = 0.05). La cuantificación de las enzimas transaminasas no mostró ningún cambio en ambos sexos de animales tratados (Cuadro 5).

Discusión

En nuestro país la medicina tradicional representa una práctica médica alternativa desde tiempos prehispánicos por razones culturales, pero además por aspectos socioeconómicos, por lo que en la actualidad su uso cotidiano es fundamental para resolver problemas de salud, principalmente en comunidades indígenas (Merino-Sánchez, 2011). Por ello es importante valorar de manera científica la eficacia e inocuidad de las plantas medicinales a través de la corroboración de sus efectos terapéuticos y su toxicología, respectivamente. Ambos aspectos son fundamentales para desarrollar estudios pre-clínicos que sustenten la investigación clínica sobre plantas con uso potencial en el tratamiento de enfermedades, principalmente aquellas relacionadas con procesos inflamatorios como la diabetes mellitus tipo 2 (44 %), enfermedad cardiovascular (35 %) y algunos tipos de cáncer como de próstata y de mama (7-41 %), entre otros (Balistreri *et al*., 2010; Hsing *et al*., 2010).

El efecto anti-inflamatorio demostrado de la especie *C. urticifolia* (Guzmán-Guzmán, 2010; Ortiz-Segura, 2011), sugiere que es un agente eficaz para prevenir la inflamación de bajo grado responsable de inducir alteraciones sobre la sensibilidad del receptor de insulina presente en el

sobrepeso y la obesidad (Bastard *et al*., 2006; González *et al*., 2006; Flehmig *et al*., 2014), y su asociación con enfermedades crónicas degenerativas.

Con base en los resultados de esta investigación, encontramos que la disminución de la ganancia de peso corporal en las ratas macho expuestas al extracto etanólico (100 y 1000 mg/kg) en el día 7 y en las ratas macho expuestas al extracto acuoso (100 y 300 mg/kg) en el día 1 y 7, no representaron signos de toxicidad y no se relacionó con el consumo de agua y alimento, encontrándose dentro de los parámetros establecidos en la curva de crecimiento de esta especie (Lewi y Marsboom, 1981). Adicionalmente, debido a que la concentración de urea del grupo de ratas hembra expuestas a 2000 mg/kg no mostró ser dosis dependiente no representa relevancia a nivel toxicológico (Mu *et al*., 2011).

Los hallazgos de esta investigación indican que la especie *C. urticifolia*, en sus extractos etanólico y acuoso, no causa mortalidad a las dosis evaluadas en ambos sexos, lo que la clasifica como una sustancia de categoría toxicológica 4 en su extracto etanólico y categoría toxicológica 5 en su extracto acuoso de acuerdo con el Sistema Mundialmente Armonizado de Clasificación (GHS; Globally Harmonized Classification System). De esta manera, se establece que la DL_{50} oral en ratas Wistar y en ambos sexos es > 1000 mg/kg para el extracto etanólico y > 5000 mg/kg para el extracto acuoso y por lo tanto el uso etno-farmacológico de la especie *C. urticifolia* es seguro en cuanto a su toxicidad aguda pues en la medicina tradicional empleada por la etnia Xi'iuy se utiliza una dosis aproximada de 0.044 mg/kg/día en su extracto etanólico para el tratamiento de una persona de 70 kg y una dosis de 0.276 mg/kg/día en su extracto acuoso. Esto representa una exposición a 22,727 y 18,115 veces menos que la DL_{50}. Sin embargo, no hay que descartar el realizar estudios posteriores de toxicidad crónica y citotoxicidad para poder continuar con la fase clínica de investigación del uso etno-farmacológico de la especie.

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Cuadro 1. Efecto del extracto etanólico de *Calea urticifolia* sobre el consumo de alimento y agua de ratas hembra y macho de la cepa Wistar, en los días 1,7 y 14 del experimento.

Dosis (mg/kg)	Consumo de alimento (g)			Consumo de agua (mL)		
	Día			Día		
	1	7	14	1	7	14
Hembras						
0	16.2±1.03	14.7±3.67	10.4±0.43	41.7±7.64	30±5.00	36.7±12.58
50	13.7±1.85	14.2±1.98	9.6±1.38	28.3±5.77	28.3±7.64	30±5.00
100	12±7.92	16±1.72	9.7±1.68	21.7±7.64	28.3±7.64	26.7±5.77
0†	17.3±3.99	16.2±1.75	17.7±3.62	48.3±16.07	43.3±5.77	56.7±5.77
300	17.6±2.02	17.8±3.78	16.9±3.12	58.3±40.72	45±21.79	51.7±25.66
1000	9.35±5.31	19.4±2.78	17.5±2.65	31.7±12.58	53.5±23.09	53.3±27.53
Machos						
0	20.6±2.94	20.5±3.81	16.3±7.12	26.4±4.76	35.7±10.18	31.4±10.69
50	18.6±1.74	20.1±2.60	12.9±2.35	27.5±2.89	35.0±4.08	28.8±4.79
100	27.8±5.23	27.6±7.15	26.6±7.17	42.5±8.66	52.5±25.66	57.5±18.03
0†	22.7±5.09	20.7±5.25	20.55±5.30	30.0±7.64	30.0±7.64	31.3±10.00
300	28.0±1.48	27.9±3.91	28.6±0.68	40.0±2.89	38.8±5.00	40.0±10.00
1000	12.5±10.57	24.2±7.73	24.4±4.26	21.3±14.43	36.3±5.00	36.3±5.77

Los valores representan la media ± DE (n = 3/grupo). Sin cambios significativos $p > 0.05$ *vs.* control. La dosis de 0 mg/kg corresponde al grupo control (agua destilada ó † aceite de maíz).

Cuadro 2. Efecto del extracto acuoso de *Calea urticifolia* sobre el consumo de alimento y agua de ratas hembra y macho de la cepa Wistar, en los días 1,7 y 14 del experimento.

Dosis	**Consumo de alimento (g)**			**Consumo de agua (mL)**		
(mg/kg)	**Día**			**Día**		
	1	7	14	1	7	14
Hembras						
0	16.2±0.95	16.7±1.92	15.8±3.59	32.5±6.45	35.0 ±4.08	35±9.13
50	20.9±4.13	18.6±2.75	20.3±0.37	36.7±11.55	38.3±7.64	40±5.00
100	16.1±1.59	18.8±0.93	16.6±2.42	23.3±11.55	31.7±2.89	31.7±2.89
300	15.8±1.16	14.7±0.74	10.9±2.08	28.3±7.64	26.7±5.77	18.3±7.64
2000	15.6±1.92	14.8±1.43	10.4±0.82	26.7±2.89	28.3±2.89	18.3±2.89
5000	5.7±9.84	18.9±1.36	20.0±3.45	18.3±18.93	40.0 ±8.66	40±5.00
Machos						
0	25.6±3.53	20.7±4.0	21.7±3.33	41.1±14.35	37.9±7.56	46.4±10.29
50	16.7±2.78	19.4±1.65	18.9±3.63	23.3±2.89	33.3±5.77	28.3±7.64*
100	14.4±0.63	18.2±1.07	18.7±1.79	25.0±8.66	30.0±0.00	31.7±2.89
300	17.4±093	20.5±1.90	21.9±1.83	31.7±2.89	30.0±0.00	33.3±2.89
2000	25.05±2.38	25.95±1.05	25.5±4.29	40.0±0.00	36.7±2.89	43.3±5.77
5000	22.5±2.75	23.7±10.4	25.9±8.23	35.0 ± 5.00	38.3 ± 17.56	41.7 ± 15.28

Los valores representan la media ± DE(n = 3/grupo). *$p \leq 0.05$ *vs.* control. La dosis de 0 mg/kg corresponde al grupo control (agua destilada).

Cuadro 3. Parámetros bioquímicos de urea, creatinina, transaminasas glutámico oxalacética (TGO) y glutámico pirúvica (TGP) de ratas hembra y macho de la cepa Wistar.

Parámetro	Hembras		Machos	
	Intervalo	**Media±DE**	**Intervalo**	**Media±DE**
Urea (mg/dL)	29-61	42.8±7.47	25-61	36.6±8.73
Creatinina (mg/dL)	0.7-1.0	0.8±0.09	0.6-0.9	0.7±0.09
TGO (U/L)	62-152	99.3±21.60	55-160	92.4±23.88
TGP (U/L)	28-104	49.4±17.31	27-100	57.6±18.25

Los valores representan intervalos del valor menor y mayor obtenidos. Y se presentan como media ± DE. n = 36

Cuadro 4. Efecto del extracto etanólico de *Calea urticifolia* sobre los parámetros bioquímicos de ratas hembra y macho al final del experimento.

	Urea (mg/dL)	**Creatinina (mg/dL)**	**TGO (U/L)**	**TGP (U/L)**
Hembras				
Agua	31.0±4.00	0.9±0.00	72.3±7.64	41.3±6.11
50 mg/kg	35.7±11.24	0.9±0.15	119.0±53.84	46.0±9.54
100 mg/kg	34.7±5.69	0.9±0.06	81.3±5.77	34.7±2.89
Aceite de maíz	50.3±16.80	0.8±0.06	127.0±51.43	61.3±8.33
300 mg/kg	39.7±2.52	0.8±0.00	111.7±27.43	51.6±11.15
1000 mg/kg	51.3±5.13	0.8±0.00	102.0±37.75	52.0±6.08
Machos				
Agua	36.8±3.40	0.8±0.06	80.8±11.32	56.5±12.56
50 mg/kg	37.2±6.70	0.7±0.05	94.5±8.23	56.8±18.17
100 mg/kg	34.0±4.36	0.8±0.06	57.0±17.06	49.3±8.02
Aceite de maíz	50.0±5.20	0.8±0.06	95.3±23.86	85.7±14.29
300 mg/kg	42.7±3.21	0.8±0.00	105.3±11.55	66.3±15.04
1000 mg/kg	49.7±9.45	0.9±0.06	86.7±4.73	81.3±9.29

Los valores representan la media ±DE (n=3/grupo). Sin cambios significativos $p>0.05$ *vs.* control (agua destilada o aceite de maíz)

Cuadro 5. Efecto de las dosis de extracto acuoso de *Calea urticifolia* sobre los parámetros bioquímicos de ratas hembra y macho al final del experimento.

	Urea (mg/dL)	**Creatinina (mg/dL)**	**TGO (U/L)**	**TGP (U/L)**
Hembras				
Agua	46.8±9.14	0.8±0.10	88.2±41.84	37.5±6.19
50 mg/kg	29.7±4.62	0.8±0.00	60.0±17.09	37.7±6.66
100 mg/kg	41.7±1.15	0.8±0.06	79.3±30.14	34.7±7.51
300 mg/kg	63.0±6.00	0.8±0.10	103.3±30.83	54.3±16.01
2000mg/kg	64.0±2.00*	0.8±0.06	90.3±17.04	49.0±7.55
5000mg/kg	60.7±8.39	0.8±0.06	89.7±26.86	55.2±13.3
Machos				
Agua	48.0±6.32	0.8±0.05	90.2±53.18	50.5±8.96
50 mg/kg	44.7±1.53	0.8±0.00	75.0±15.00	47.0±2.65
100 mg/kg	41.7±3.79	0.8±0.00	72.3±15.57	54.7±18.50
300 mg/kg	42.3±5.13	0.8±0.06	69.0±43.3	40.7±9.50
2000mg/kg	53.2±14.36	0.8±0.05	104.5±29.03	84.5±43.31
5000mg/kg	48.7±16.50	0.7±0.06	118.3±27.15	40.0±28.62

Los valores representan la media ± DE (n = 3/grupo). *$p \leq 0.05$ *vs* control (agua destilada).

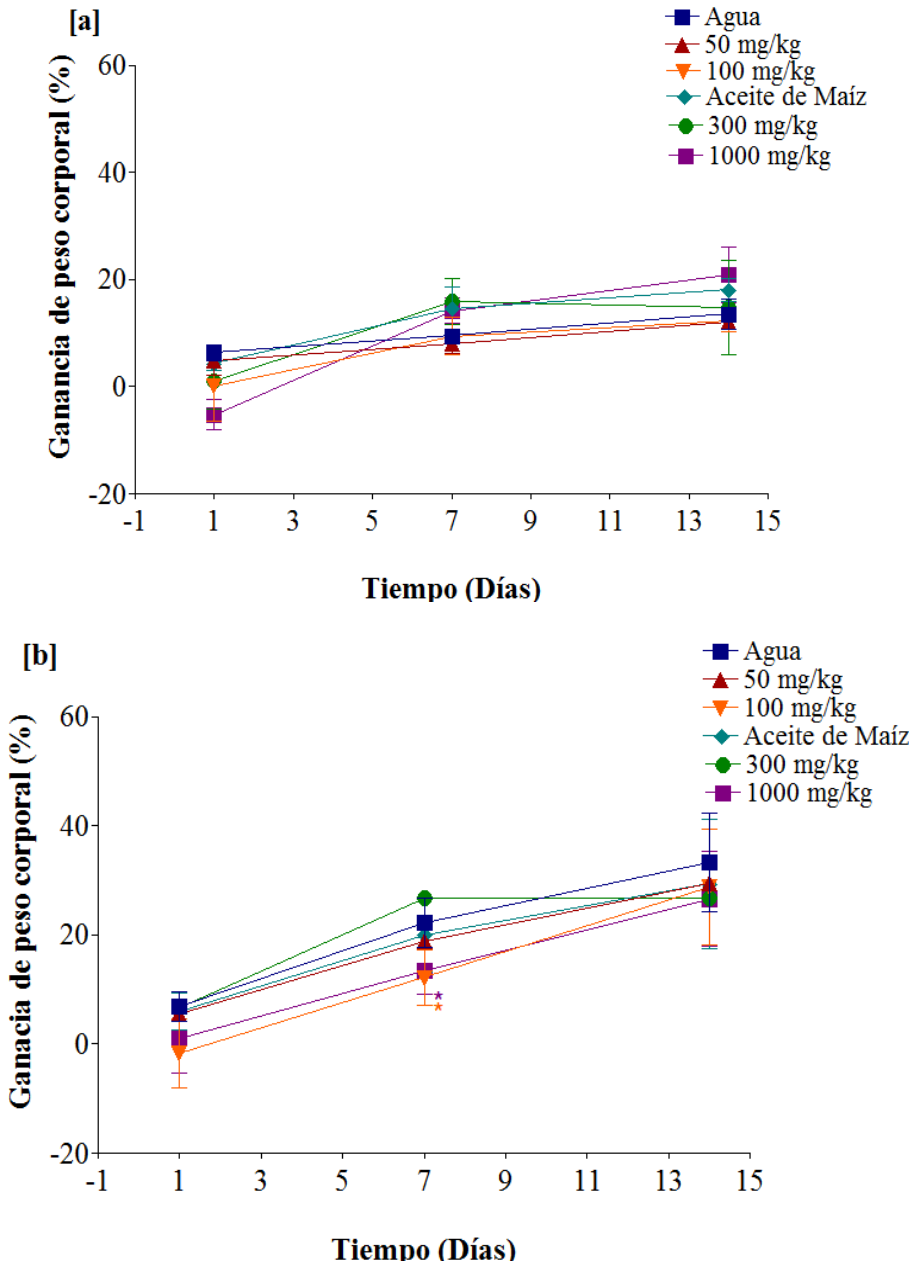

Figura 1. Ganancia de peso corporal (%) en ratas hembra (**a**) y macho (**b**) de la cepa Wistar en los días 1, 7 y 14 posterior a la administración oral del extracto etanólico de *Calea. urticifolia*. Los puntos representan la media ± DE (n = 3/grupo). *$p \leq 0.05$ *vs.* control (agua destilada o aceite de maíz).

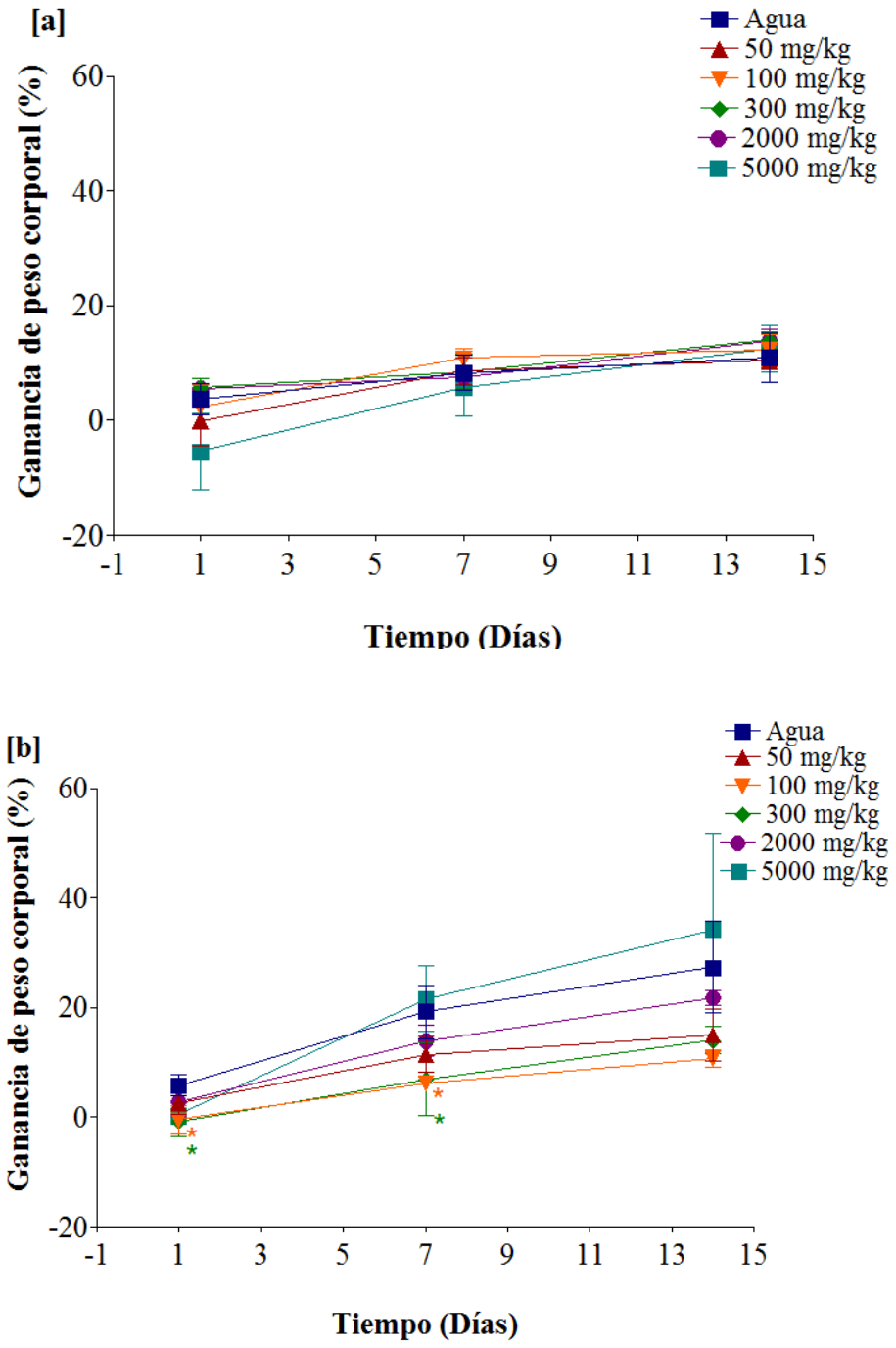

Figura 2. Ganancia de peso corporal (%) en ratas hembra (**a**) y macho (**b**) de la cepa Wistar en los días 1, 7 y 14 posterior a la administración oral del extracto acuoso de *Calea urticifolia*. Los puntos representan la media ± DE (n = 3/grupo). *$p \leq 0.05$ *vs.* control (agua destilada).

Capítulo II.

Sub-chronic oral toxicity of *Calea urticifolia* lyophilized aqueous extract in Wistar rats

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ABSTRACT

Ethnopharmacological relevance: Calea urticifolia is a shrub of the Asteraceae family and is used by the Xi'iuy ethnic group of San Luis Potosi, Mexico. It is widely distributed from Mexico to Panama in semi-warm and warm climates, located in tropical and oak forests. The tea prepared with the leaves is used as a remedy to treat gastric ulcers and diabetes and is an anti-inflammatory agent. Despite its traditional use, there are no reports on the sub-chronic toxicity of the aqueous extract of *C. urticifolia*.

Aim of the study: To evaluate the *in vivo* sub-chronic toxicity of *C. urticifolia* lyophilized aqueous extract (CuAqE) as part of its preclinical evaluation.

Materials and methods: Male and female young adult Wistar rats (180-200 g) were treated with 0.5, 50 and 500 mg/kg of CuAqE by gavage during 28 days. The mortality incidence, clinical signs and body-weight gain were registered every day. The Organs weight (liver,

heart, lungs, stomach, esophagus, spleen, kidneys, brain and cerebellum), the biochemical (urea, creatinine, alanine transaminase and aspartate transaminase) and hematological (white blood cells, red blood cells, platelets, hematocrit, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin) parameters were evaluated at the end of study.

Results: Sub-chronic oral administration of CuAqE for 28 days did not produce mortality or clinical signs. Food and water consumption, organs weight, biochemical and hematological parameters showed not-significant changes in both male and female rats ($p > 0.05$). Body weight gain was significantly decrease when 50 mg/kg was administered for 21 and 28 days compared with the control group (28 ± 25 and 44 ± 25 %, respectively) and with 500 mg/kg for 7, 14, 21 and 28 days(95 ± 66, 64 ± 45, 59 ± 31, 64 ± 19%, respectively) in female rats ($p < 0.05$).

Conclusion: Results of this study showed that the CuAqE had a low grade of toxicity. Therefore *C. urticifolia* tea at traditional dose (0.55 mg CuAqE/kg body weight) can be safely consumed.

Keywords: Calea urticifolia, sub-chronic toxicity, preclinical study

1. Introduction

World Health Organization (WHO) estimates that 80% of the population in developing countries uses the traditional medicine as primary health care and sometimes it is the only source of care. In addition, some populations use the traditional medicine because they think that "natural" is synonym of innocuous (WHO, 2013).

Calea urticifolia commonly named as "negrito" is a plant used as a tea prepared with *C. urticifolia* leaves by the Xi'iuy ancient native community of San Luis Potosí, México, to treat diabetes and gastric ulcers and as an anti-inflammatory agent (Guzman, 2010). The

chemical analysis of organic extracts of *C. urticifolia* leaves showed the presence of sesquiterpene lactones with germacrane skeleton (Bohlmann, 1979; Yamada *et al*., 2004). These compounds have shown different biological activities such as inhibition of melanin biosynthesis in mouse B16 melanoma cells (Ohguchi *et al*., 2009), apoptosis induction in HL60 cells (Nakagawa *et al*., 2005), anti-oxidant effect in PC12 pheochromocytoma cells (Umemura *et al*., 2008) and suppressive activity against adipocyte differentiation (Matsuura *et al*., 2005).

Preliminary phytochemical studies have determinate that the aqueous extract of *C. urticifolia* (CuAqE) contains carbohydrates, flavonoids, Tannins and catechins (Zermeño, 2013). Furthermore, the phenolic compounds profile was characterized recently where a mix of caffeoyl-quinic acid and flavonoid glycoside were found (Torres, 2015). These compounds present anti-oxidant, anti-inflammatory, analgesic, and anti-cancer activities (Upadhyay & Dixit, 2015; Puangpraphant *et al*., 2011; dos Santos *et al*., 2010; Baraldi *et al*., 2008; Han et al., 2008; Lai *et al*., 2007).

Recently, we evaluated the anti-inflammatory and the anti-oxidant effects of the CuAqE in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. These results showed that the phytochemical compounds present in the CuAqE were able to modulate NF-κB signaling pathway through suppressing pro-inflammatory markers such as inducible nitric oxide synthase (iNOS), cytokines and chemo-attractant molecules (Torres, 2015). Despite of these relevant findings, is important to perform toxicity studies of CuAqE to ensure the health of the populations that consume it. Therefore, the objective of this study was to evaluate the *in vivo* sub-chronic toxicity of the lyophilized aqueous extract of *C. urticifolia* as part of its pre-clinical evaluation.

2. Materials and methods

2.1. Herbal material

Fresh leaves of adult plants of *C. urticifolia* were collected in Xi'iuy ancient native community of Potrero del Carnero San Luis Potosi, Mexico in August 2012. Herbal material was dried in absorbent paper and stored at room temperature until use.

2.2. Preparation of C. urticifolia lyophilized aqueous extract

Dry leaves of *C. urticifolia* were milled before extraction. Ten grams were transferred to 100 mL of distilled water and boiled for 5 min. Once the aqueous extract was cooled down, it was filtered and freeze-dried (Freeze-dryer TFD5505, Ilshin®). The yield was 14.8% of the residue which was stored at 4˚C until use.

2.3. *Experimental animals*

Male and female healthy adult young Wistar rats weighing 180-200 g were used. The animals were housed in individual acrylic cages at temperature of 23 ± 3˚ C with a 12:12 light/dark cycles. Rats were provided with standard rodent food (Formulab Chow 5001) and water *ad libitum* during adaptation (5 days) and the experimental time (28 days). The animals were obtained from the College of Medicine of the Autonomous University of San Luis Potosi, Mexico and were handled following the Mexican Norm for Animal Care and Handing (Norma Oficial Mexicana NOM-062-ZOO-1999).

2.4. Sub-chronic toxicity

Sub-chronic toxicity was tested following the Guideline of the Organization for Economic Cooperation and Development number 407 (OCDE, 2008). Briefly, forty rats (20 male and 20 female) were randomly divided in four groups (5 rat/sex group). The first

group received distilled water and was considered as the negative control, the other three groups were administered with doses of 0.5, 50 or 500 mg/kg of CuAqE, respectively. All treatments were orally-administrated daily using an intra-gastric probe (< 0.5 mL) for 28 days. During administration period, the animals were observed for general behavior, toxic manifestations and mortality daily. Body weight changes and food and water consumption were recorded during 28 days. Body weight was evaluated at days 1, 7, 14, 21 and 28 days, food and water consumption was reported as the accumulated consumption for each seven days. At the end of the experimental time, on day 30, a sample of blood was collected by cardiac puncture to perform the hematological parameters and the biochemical analysis. After the blood collection, all animals were euthanized by overdose of chloroform and the liver, heart, lungs, stomach, esophagus, spleen, kidneys, brain and cerebellum were obtained and weighted.

2.5. Biochemical and hematological measurement

The biochemical parameters, such as creatinine, urea, alanine transaminase (ALT) and aspartate transaminase (AST) were analyzed in blood using a RA-50 Bayer instrument. Creatinine concentration was quantified using the kinetic-colorimetric method (Jaffé) at 492 nm (Perazzi and Angerosa, 2011) and urea was quantified using the GLDH urease-kinetic method at 340 nm (Spinreact). ALT and AST were determinate by NADH kinetic-UV method at 340 nm.

The hematological parameters such as the total red cells count (RBC), white blood cells count (WBC), platelets, hematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV), and mean haemoglobin concentration (MCH) were determined using a blood auto-analyzer (Mindray BC-3200).

2.6. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Parametric data were analyzed by one-way analysis of variance (ANOVA) and statically significance among groups was determinate using a*post hoc* Tukey's test. For all cases, a p value < 0.05 was considered statistically significant.

3. Results

3.1. Sub-chronic oral toxicity

3.1.1. Effect of sub-chronic oral administration of CuAqE on general behavior and mortality

Sub-chronic oral administration of CuAqE for 28 days did not produce changes in the general behavior, toxicity signs or clinical manifestations in both female and male Wistar rats. Thus, the no-observed-adverse-effect-level (NOAEL) was higher than 500 mg/kg. Moreover, the CuAqE did not produce mortality when the extract was administrated orally at 0.5, 50 or 500 mg/kg in both female and male rats.

3.1.2. Effect of sub-chronic oral administration of CuAqE on Body weight gain and food and water consumption

Significant changes were observed in the body weight gain compared with the control group (Fig. 1a, $p < 0.05$), which was lower at 50 mg/kg for 21 and 28 days (28 ± 25 and 44 ± 25%, respectively) and at the dose of 500 mg/kg administered for 7, 14, 21 or28 days (95 ± 66; 64 ± 45; 59 ± 31 and 64 ± 19%, respectively). No significant changes were observed on body weight gain in male rats after 1, 7, 14, 21 or 28 days of administration of CuAqE

(Fig. 1b). Moreover, there were no significant changes in food and water accumulated consumption (each7 days) when they were compared with the treated groups (0.5, 50 and 500 mg/kg) in both female and male rats (Fig. 2a, 2c and Fig. 2b, 2d, respectively).

3.1.3. Effect of sub-chronic administration of CuAqE on the biochemical and hematological parameters

The effect of sub-chronic oral administration of CuAqE (0.5, 50 and 500 mg/kg) in female and male Wistar rats during 28 days, did not produce significant changes in the biochemical parameters (urea, creatinine, AST and ALT) (Table1). Moreover, there were no significant alterations on any of the hematological parameters in both genders after 28 days of CuAqE treatment (Table 2).

3.1.4 Effect of sub-chronic administration of CuAqE on organs weight

Table 3 shows the weight of organs of female and male Wistar rats after the administration of CuAqE at 0.5, 50 and 500 mg/kg during 28 days and organs weight were no significantly modified when the values were compared with the control group.

4. Discussion

Traditional medicine is used across the world in low and high income countries; the World Health Organization (WHO) estimates that about 80% of the world's population uses traditional medicine as either the main source of health care or as a complement to it (WHO, 2013). However, despite that medicinal plants are widely used, not all them have been evaluated on their toxicological effects and health risks for the oral use. Therefore, experimental studies on health risks of the oral usage of these plants are necessary. In this

research we evaluated the sub-chronic toxicity of CuAqE to contribute with its toxicological evaluation and to validate its safe use as part of its pre-clinical evaluation.

Currently, to estimate the safety of drugs and plants, the toxicological studies have been carried out in different experimental rodent animals. Adverse effects on toxicity studies in rats have showed a good correlation with humans in hematological, gastrointestinal and cardiovascular effects, moreover these studies have been useful to estimate safe doses of medicinal plants in humans (Olson *et al*., 2000).

Toxicity studies include acute and chronic evaluations among others. Previous results about the oral acute toxicity of CuAqE showed a LD_{50} > 5g/kg which, in agreement with the Global Harmonized Classification System, was classified as a substance of category 5; this is to say as a substance of low toxicity (Torres *et al*., 2016). In that study we evaluated an acute single dose of CuAqE, those results were useful to select proper doses of the CuAqE (0.5, 50and 500 mg/kg) to evaluate the sub-chronic oral toxicity in Wistar rats (both genders) through of the daily oral administration.

In this sub-chronic oral toxicity study, the results showed no mortality, no changes in general behavior and no altered clinical-signs with any doses used. In addition, CuAqE did not produce significant changes in both food and water consumption indicating no effect in normal appetite, this results are in agreement with no changes in this parameters in *Artemisa afa* a species of Asteraceae family and with others such as *Pterocarpus soyauxii* Taub (Papilonaceae) and *Poligala fructicosa* (Poligalaceae) (Mukinda and Syce, 2007, Tchamadeu *et al*, 2011; Mukinda and Eagles, 2010). However, in female rats, body weight gain was lower than control group at dose of 50 mg/kg at 21 and 28 days and by 500 mg/kg at 7, 14, 21 and 28 days. Although, a decrease in body weigh has been used as an indicator of adverse effect in toxicity studies (Teo *et al*., 2002; El Hilaly *et al*., 2004), in

this study the no-body weight gain was not correlated with a decreased appetite, therefore, this effect was considered no toxic adverse effect, because the body weight reached in the female rats was into the normal growth curve for Wistar rats (Lewi y Marsboom, 1981).

Moreover, hematological (RBC, WBC, Hct, Hb, MCV and MCH) and biochemical (urea, creatinine, AST and ALT) parameters in sub-chronic toxicity studies are important to show abnormal behavior in metabolic processes and in the general health state. In addition these studies can reveal alterations of the hematological system and adverse effects in kidney and liver function, which can be related with toxic effects, body injury, malnutrition and stress (Rhiouani *et al*, 2008). In the present study, the administration of CuAqE for 28 days did not produce alterations in hematological and biochemical parameters suggesting no alterations in vital functions and toxic effect (Tahraoui *et al*, 2010).

Finally, our findings provide relevant information to support the safety of oral administration of CuAqE. And moreover, these results can be useful to validate more pre-clinical studies and support further clinical trials. In addition, this research contributes to establish and reinforce the category of low toxicity of the *Calea urticifolia* plant.

5. Conclusion

Sub-chronic oral administration of the CuAqE (0.5, 50, and 500 mg/kg) did not produce death, behavioral changes or sign of toxicity. Moreover, the administration of the CaAqE did not produce changes on food and water consumption, hematological or biochemical parameters and weight of organs contributing to the classification of non-toxicity of the plant. Therefore traditional doses typically used by the Xi'iuy community of *C. urticifolia* (0.55 mg/kg) can be consumed safely.

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Table 1. Effect of oral administration of CuAqE for 28 days on urea, creatinine, AST and ALT in female and male Wistar rats.

Dose	Urea mg/dL	Creatinine mg/dL	AST U/L	ALT U/L
Female rats				
Control	39.0 ± 5.10	0.92 ± 0.045	94.6 ± 39.48	71.6 ± 20.86
0.5 mg/kg	39.4 ± 5.50	0.92 ± 0.045	107.8 ± 28.52	75.4 ± 12.14
50 mg/kg	37.6 ± 3.85	0.88 ± 0.045	84.0 ± 28.08	74.4 ± 24.64
500 mg/kg	35.0 ± 10.27	0.88 ± 0.045	122.4 ± 19.45	81.8 ± 18.85
Male rats				
Control	35.4 ± 4.16	0.86 ± 0.055	119.4 ± 13.26	82.0 ± 28.03
0.5 mg/kg	33.0 ± 4.30	0.86 ± 0.055	101.4 ± 26.59	89.0 ± 34.32
50 mg/kg	35.4 ± 16.95	0.86 ± 0.055	102.0 ± 18.10	84.0 ± 22.76
500 mg/kg	32.2 ± 6.06	0.84 ± 0.055	178.6 ± 92.67	97.4 ± 26.33

Values represent mean ± SD (n=5/group). *$p < 0.05$ *vs.* control (Distilled water).

Table 2. Effect of CuAqE on hematological parameters of female and male Wistar rats after administration of CuAqE for 28 days.

	Hemoglobin g/dL	**Hematocrit %**	**Red cells /mm³**	**MCV µ³**	**MCH g/dL**	**Platelets /mm³**	**White cells /mm³**
Female rats			($x10^6$)			($x10^5$)	($x10^3$)
Control	16.1±4.3	42.6±11.1	7.4± 1.8	57.0±2.3	37.8±0.7	8.6±1.2	6.9±3.6
0.5 mg/kg	17.2±0.9	47.0±2.6	8.3± 2.9	56.6±2.0	36.6±0.3	4.9±4.5	8.4±2.7
50 mg/kg	17.9±1.6	49±4.6	8.6± 6.5	57.1±1.8	36.6±0.8	8.3±1.3	8.0±2.8
500 mg/kg	16.6±0.6	45.1±1.3	7.8± 4.7	58.3±2.2	36.8±0.8	8.3±0.6	6.9±2.0
Male rats							
Control	18.5±1.23	51.2±3.2	8.9± 5.7	57.6±0.4	36.1±0.5	7.0 ± 0.8	8.2±2.4
0.5 mg/kg	15.9±2.96	47.9±7.5	7.1± 2.1	71.3±18.8	33.4±4.0	5.9 ± 2.3	8.9±0.8
50 mg/kg	17.2±2.16	49.8±4.3	6.7± 1.7	78.1±19.7	34.5±2.3	5.4 ± 1.9	7.7±2.2
500 mg/kg	16.9±1.84	46.7±4.4	8.4± 1.1	55.8±2.4	36.2±0.7	8.1 ± 3.0	8.3±2.0

Values are mean ± SD (n=5/group). *$p < 0.05$ *vs.* control (Distilled water).

Table 3. Effect of CuAqE on weight of different organ of female and male Wistar rats after administration of CuAqE for 28 days.

	Liver	Heart	Lungs	Stomach	Esophagus	Spleen	Left kidney	Right kidney	Brain	Cerebellum
Female rats										
Control	7.2±0.5	0.76±0.1	1.25±0.1	1.15±0.1	0.15±0.02	0.51±0.1	0.81±0.06	0.88±0.04	1.24±0.04	0.44±0.04
0.5 mg/kg	8.0±0.6	0.82±0.1	1.22±0.1	1.26±0.1	0.15±0.02	0.52±0.0	0.92±0.10	0.98±0.11	1.23±0.05	0.42±0.04
50 mg/kg	7.4±0.4	0.76±0.1	1.20±0.1	1.26±0.1	0.16±0.01	0.47±0.0	0.78±0.11	0.80±0.08	1.23±0.03	0.46±0.03
500 mg/kg	7.6±0.7	0.84±0.1	1.20±0.1	1.21±0.2	0.16±0.01	0.51±0.1	0.85±0.11	0.91±0.12	1.18±0.04	0.44±0.05
Male rats										
Control	10.3±0.8	1.06±0.2	1.44±0.1	1.41±0.1	0.19±0.03	0.56±0.1	1.14±0.1	1.13±0.09	1.33±0.04	0.45±0.05
0.5 mg/kg	11.5±0.9	1.07±0.2	1.31±0.2	1.40±0.1	0.17±0.03	0.53±0.1	1.26±0.1	1.21±0.19	1.32±0.08	0.47±0.03
50 mg/kg	11.0±1.1	0.97±0.1	1.34±0.1	1.39±0.1	0.19±0.03	0.56±0.0	1.15±0.0	1.20±0.15	1.36±0.06	0.46±0.07
500 mg/kg	9.4±1.9	1.03±0.1	1.32±0.3	1.27±0.2	0.15±0.03	0.53±0.1	1.05±0.24	1.03±0.23	1.26±0.07	0.42±0.03

Values are mean ± SD (n=5/group)* $p < 0.05$ *vs* control.

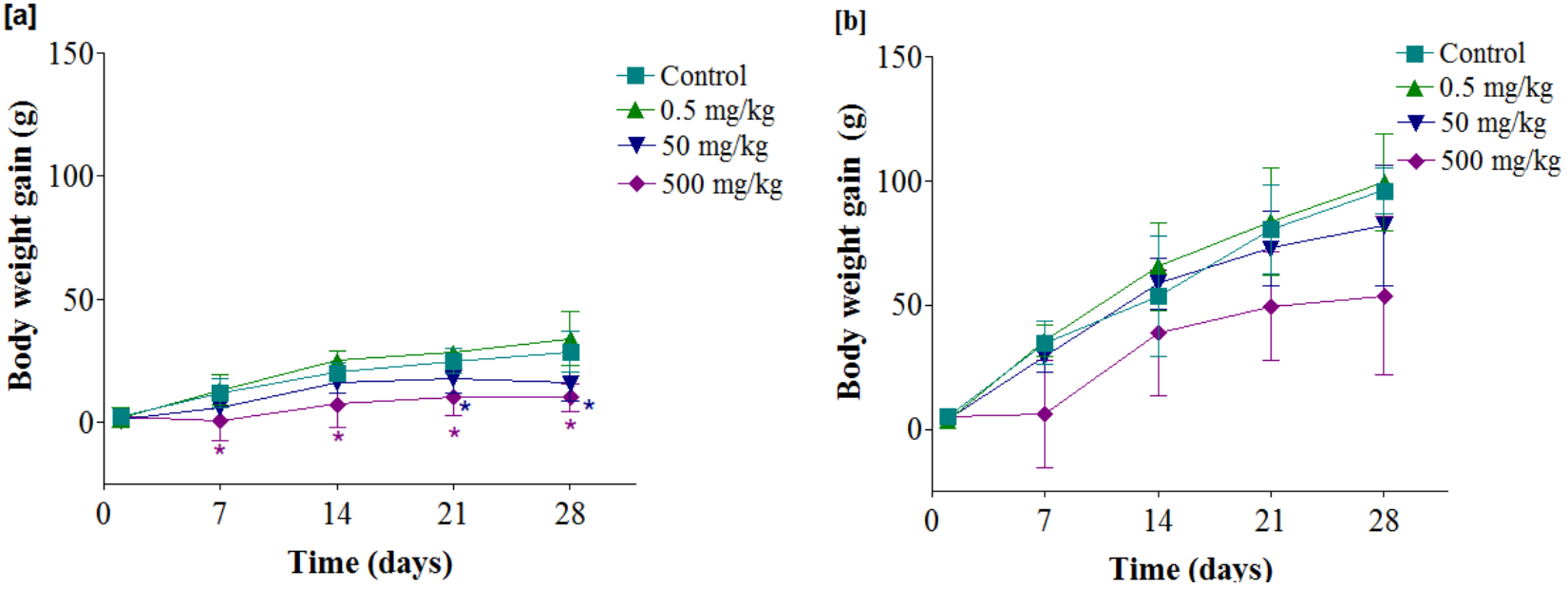
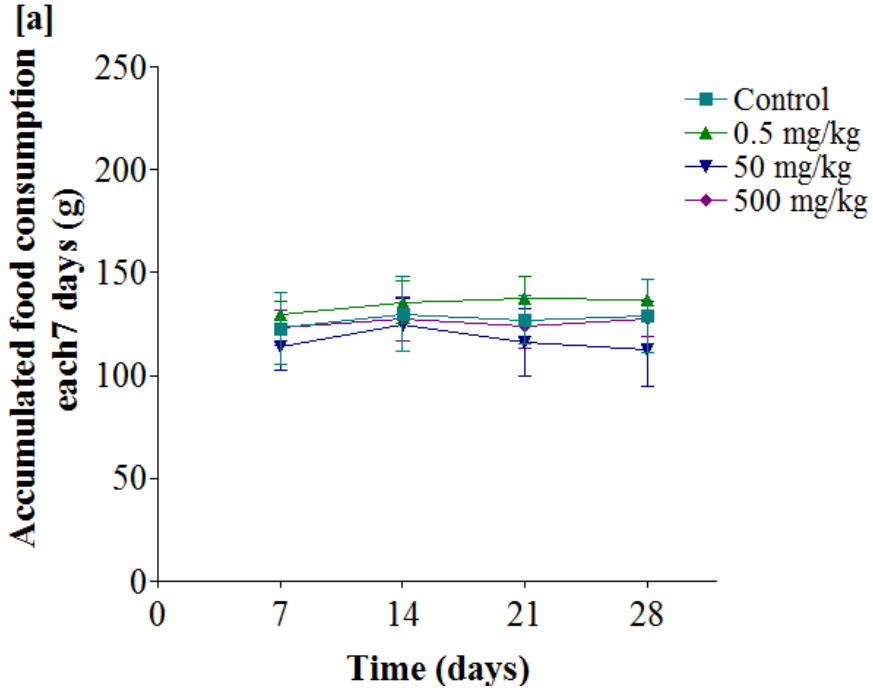
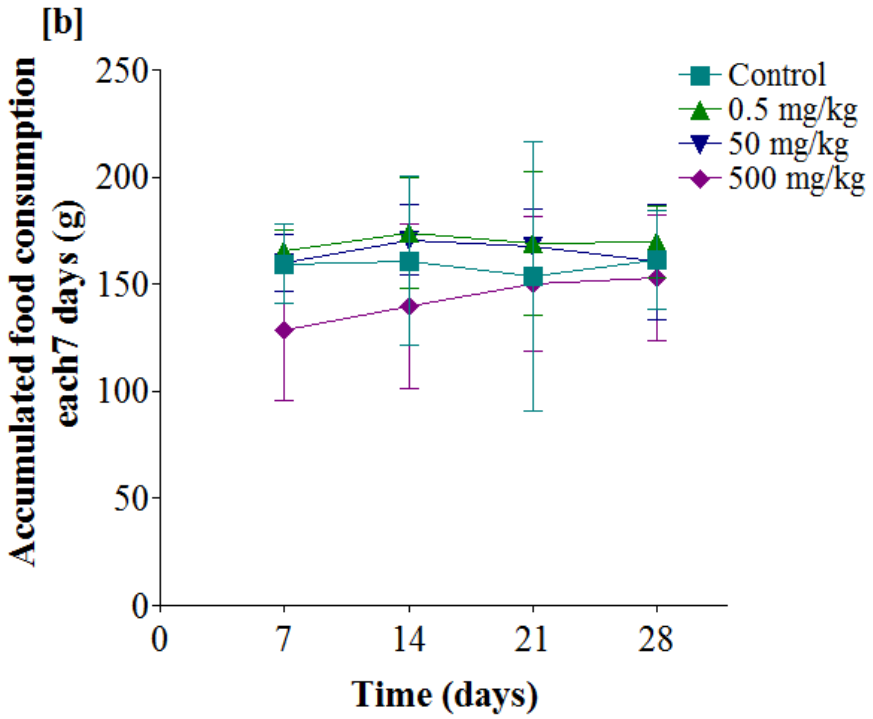


Fig. 1. Effect of oral administration of the CuAqE on body weight gain in female (**a**) and male (**b**) Wistar rats at 1, 7, 14, 21 and 28 days. The points represent the mean ± SD (n=5/group). *$p < 0.05$ *vs.* control/day.


[a]
Accumulated food consumption each7 days (g)
250
200
150
100
50
0
0
7
14
21
28
Time (days)
Control
0.5 mg/kg
50 mg/kg
500 mg/kg


[b]
Accumulated food consumption each7 days (g)
250
200
150
100
50
0
0
7
14
21
28
Time (days)
Control
0.5 mg/kg
50 mg/kg
500 mg/kg

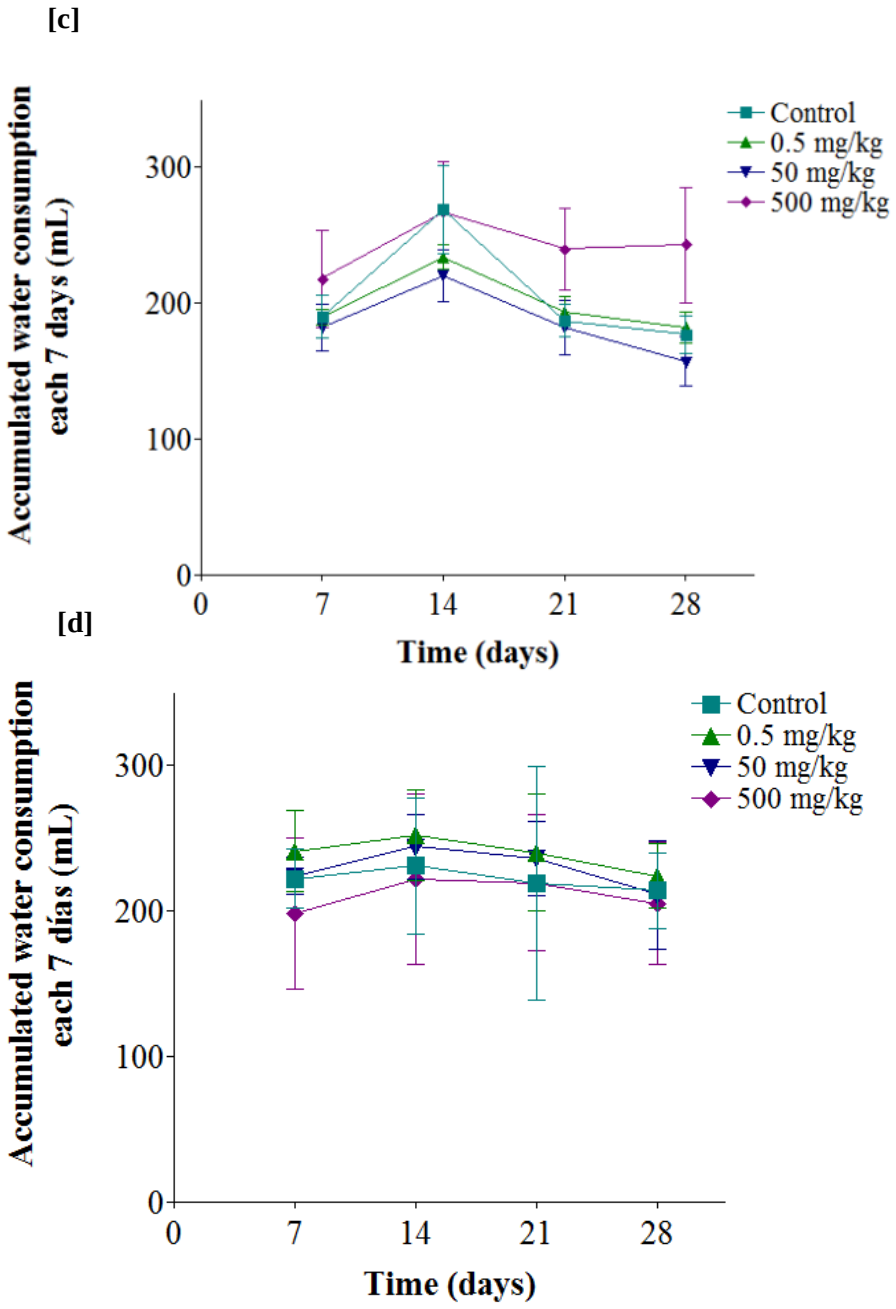


Fig. 2. Effect of sub-chronic oral administration of the CuAqE on accumulated consumption of food and water in female (**a, c**) and male (**b, d**) Wistar rats at 7, 14, 21 and 28 days. The points represent the mean ± SD (n=5/group). *$p < 0.05$ *vs.* control/day.

Capítulo III.

Anti-inflammatory and anti-oxidant effect of *Calea urticifolia* lyophilized aqueous extract on lipopolysaccharide-stimulated RAW 264.7 macrophages through suppressing nuclear factor NF-κB signaling pathway

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ABSTRACT

Ethnopharmacological relevance: *Calea urticifolia* leaves are traditionally used as a remedy to treat gastric ulcers, diabetes and inflammation by the Xi'iuy ancient native community of San Luis Potosi, Mexico.

Aim of the study: To identify phenolic compounds of *C. urticifolia* lyophilized aqueous extract (CuAqE) and evaluate *in vitro* its anti-inflammatory and anti-oxidant properties in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

Materials and methods: Phenolic compounds were analyzed by LC-ESI-MS; RAW 264.7 macrophages were stimulated with 1 µg/mL of LPS and treated with 10, 25 50, 75 and 100

µg/mL of CuAqE. Nitric oxide (NO) release, tumor necrosis factor (TNF)-*a*, prostaglandin E_2 production, inducible NO synthase (iNOS), cyclooxygenase (COX)-2, nuclear factor-κB (NF-κB) p65, NF-κB p50 expression and reactive oxygen species (ROS) were quantified, others pro-inflammatory proteins were evaluated with a membrane antibody array.

Results: A mix of caffeoyl-quinic acid derivatives and flavonoid-glycosides was found in the CuAqE. The inflammation was inhibited by the CuAqE by suppressing the iNOS/NO pathway through the inhibition of the NF-κB p65 and p50sub-units. The production of ROS was inhibited in a dose-dependent manner in LPS-stimulated macrophages. Moreover, the expression of inflammatory markers was suppressed (34.5 to 88.3%) by the treatment with the CuAqE.

Conclusion: Phenolic compounds in the CuAqE, such as caffteoyl-quinic acid derivatives and flavonoid-glycosides, could be responsible to inhibit LPS-induced inflammation and oxidative stress in RAW 264.7 macrophages by suppressing NF-κB pathway. Therefore, these results support the traditional knowledge of *C. uritifolia* tea such as an anti-inflammatory and antioxidant agent.

Keywords: Calea urticifolia, inflammation, phenolic compounds, nitric oxide, reactive oxygen species, NF-κB.

1. Introduction

In recent decades, a chronic low-grade inflammation has been widely related with metabolic disorders and chronic diseases, such as insulin resistance, obesity, type 2 diabetes (T2D), cardiovascular diseases and cancer (Xu *et al*., 2015; Anderson *et al*., 2014; Faloia, 2012; Balistreri *et al*., 2010; Gustafson, 2010; Tilg & Moschen, 2008; Hotamisligil, 2006).

Chronic low-grade inflammation is characterized by an increased infiltration of activated macrophages in dysfunctional adipose tissue with persistent release of pro-inflammatory markers including nitric oxide (NO), cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and adipokines as adiponectin, leptin and resistin, among others. Furthermore, several transcription factors and kinases such as c-Jun N-terminal kinase (JNK) and inhibitor of kappa B kinase (IKK B) and nuclear factor-κB (NF-κB) are involved in this process (Tilg & Moschen, 2008). Moreover, the NF-κB signaling pathway represents a pivotal role in developing chronic low grade inflammation-related diseases. Therefore, therapeutic compounds targeting NF-κB signaling pathway could contribute to inhibit this inflammatory process (de Mejia *et al*., 2013).

Traditional medicine is an important natural source of phytochemical compounds with large therapeutic effects and represents the main health resource to many populations (Cordell, 2014). The World Health Organization (WHO) estimates that 80% of the population from developing countries uses traditional medicine as their primary health care (WHO, 2013).

Calea urticifolia is a medicinal plant used by the Xi'iuy ancient native community of San Luis Potosi, Mexico and is widely distributed from Mexico to Panama in semi-warm and warm climates, located in tropical and oak forests. It is a shrub of 1-3 m high with yellow flowers that belongs to the Asteraceae (Compositae) family; its common names are negrito, hierba del negro, jaral de castilla, chilchaca and juanislama. The tea prepared with its leaves is used traditionally as a remedy to treat gastric ulcers, diabetes and inflammatory processes (Guzman, 2010). Previous studies have determinate that *C. uriticifolia* contains sesquiterpene lactones with germacrane skeleton (Yamada et al., 2004). These compounds are characteristic and predominant in species of the Asteraceae family which have potent

therapeutic effects (Camilotti *et al*., 2014). Sesquiterpene lactones isolated from *C. urticifolia* have shown different biological activities such as inhibition of melanin biosynthesis in mouse B16 melanoma cells (Ohguchi *et al*., 2009), apoptosis induction in HL60 cells (Nakagawa *et al*., 2005), anti-oxidant effect in PC12 pheochromocytoma cells (Umemura *et al*., 2008) and suppressive activity against adipocyte differentiation (Matsuura *et al*., 2005). However, studies about the phenolic compounds and the molecular mechanism of action of the CuAqE are limited. Therefore, the objective of this research was to characterize the phenolic compounds from the CuAqE, and evaluate their anti-inflammatory and anti-oxidant activity as well as the signaling pathways related with these effects in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

2. Material and methods

2.1. Materials

RAW 264.7 murine macrophages cell line and Dulbecco's modified Eagle's medium with L-glutamine (DMEM) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Fetal bovine serum and mouse TNF-α ELlSA assay (KMC3011) were purchased from Invitrogen (Grand Island, NY, USA). Penicillin (1000 U/mL), streptomycin (1000 U/mL), sodium pyruvate (100 mM), sodium nitrite, sulfanilamide, N-1-(naphthyl) ethylene-diamine-diHCl, and LPS from *Escherichia coli* (O55:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell Titer 96® AQueous One Solution Proliferation Assay kit (MTS/PES) was purchased from Promega (Madison, WI, USA). Mouse monoclonal antibodies for cyclooxygenase (COX)-2 (sc19999), actin (sc8432) and rabbit polyclonal antibody IgG anti-Glyceraldehyde 3-

phosphate dehydrogenase (GAPDH)-horseradish peroxidase (HRP) conjugate (sc25778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal antibody for inducible nitric oxide synthase (iNOS) (ab-49999), rabbit monoclonal antibodies against NF-κBp105/p50 (ab-32360) and NF-κBp65 (ab-32536) were purchased from Abcam Inc. (Cambridge, MA, USA). Anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibody was obtained from GE Healthcare (Buckinghamshire, UK). Prostaglandin E-2 (PGE_2) EIA monoclonal assay was purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Herbal material.

Fresh leaves of adult plants of *C. urticifolia* were collected in Xi'iuy ancient native community of Potrero del Carnero San Luis Potosi, Mexico in August 2012. Herbal material was separated and transferred to absorbent paper for drying and stored at room temperature until use.

2.3. Preparation of C. urticifolia lyophilized aqueous extract.

Dry leaves of *C. urticifolia* were milled before extraction. Ten grams were transferred to 100 mL of distilled water and boiled for 5 min. Once the aqueous extract was cooled down, it was filtered and freeze-dried (Freeze-dryer TFD5505, Ilshin®, Hialeah, FL, USA). The yield was 14.8% of the residue which was stored at 4˚C until use.

2.4. Phenolic compounds LC-ESI-MS Analysis

The lyophilized aqueous extract of *C. urticifolia* was extracted with methanol (1:15 w/v) and samples were analyzed in a Thermo Electron LTQ Orbitrap Discovery Mass

Spectrometer, a linear ion trap (LTQ XL) MS, coupled to a high precision electrostatic ion trap (Orbitrap) MS with a high energy collision (HCD) cell. It also contains an Ion Max electro spray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system (ACCELA 1250 UHPLC pump, ACCELA1 HTC cool stack auto-injector, and a ACCELA 80 Hz PDA detector); all performing under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software. The MS was analyzed with the ESI probe in the negative mode. The source inlet temperature was 300°C, the sheath gas rate was set at 50 arbitrary units, the auxiliary gas rate was set at 5 arbitrary units, and the sweep gas rate was set at 2 arbitrary units. The maximal mass resolution was set at 30,000, the spray voltage was set at 3.0 kV and the tube lens was set at -100 V. Other parameters were determined and set by the calibration and tuning process. The column was a 3 x 150 mm Inertsil reverse phase C-18, ODS 3, 3 µm column (Metachem, Torrance, CA, USA). The initial solvent system was 20% methanol and 80% water with 0.25% formic acid at a flow rate of 0.25 mL per min. After injection (15 µl), the column was worked out with a linear gradient to 100% methanol over 50 min. The column effluent was monitored at 280 nm in the PDA detector. The software package was set to collect mass data between 150-1000 AMUs. Generally, the most significant sample ions produced under these conditions were $[M^{-}1]^{-}$and $[M^{+}HCOO]^{-}$ (Puangpraphant *et al,* 2010).

2.5. Cell viability assay in RAW 264.7 macrophages

Cell viability assay was performed using the Cell Titer 96® AQueous One Solution Proliferation Assay. Briefly, $1x10^4$ cells were seeded in a 96-well plate in a total volume of 200 µL with DMEM medium and were incubated at 37°C in 5% CO_2 atmosphere for 24 h. After the incubation, the medium was replaced; the cells were treated with the CuAqE (10,

25, 50, 75 and 100 µg of CuAqE/mL in DMEM culture medium) and incubated for an additional 24 h. After this time, the medium was removed and replaced with 100 µL of fresh medium and 20 µL of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) were added to each well and incubated for 2 h at 37°C in 5% CO_2. Finally, the absorbance was determined at 515 nm in an Ultra Micro plate Reader (Biotek Instruments, Winooski, USA). Percentage of viable cells was calculated with respect to the group of untreated cells (100 % of viability).

2.6. RAW 264.7 macrophages cell culture

RAW 264.7 macrophage culture was done seeding 2 x 10^5 cells in a six-well plate and was incubated at 37°C in 5 % CO_2 atmosphere for48 h. After the incubation, the cells were stimulated with 1 µg/mL LPS, treated with different concentrations of CuAqE (10, 25, 50, 75 y 100 µg/mL) and incubated for 24 h. Finally, the spent medium was collected and analyzed for NO, TNF-α and PGE_2 production. Cell lysates were used for Western blot analysis of iNOS and COX-2 expression.

2.7. Nitric oxide release

Nitric oxide (NO) release was quantified as nitrite production using Griess reaction. Briefly, 100 µL of spent medium was added to a 96-well plate with an equal amount of Griess reagent (1% sulfanilamide and 0.1 % N-1-(napthyl) ethylenediamine-diHCl in 2.5% H_3PO_4). The plate was incubated for 5 min at room temperature and the absorbance was evaluated at 550 nm in an Ultra Microplate Reader (Biotek Instruments, Winoski, USA). The amount of NO released was calculated using a sodium nitrite standard curve ($y = 0.066x + 0.071$, $R^2 = 0.998$).

2.8. Tumor necrosis factor alpha and Prostaglandin E_2 measurement

The production of TNF-α and PGE_2 was quantified in the cell culture supernatant using commercially available assays (Life Science Technologies and Cayman Chemical, respectively). The procedure was done following the manufacturer's instructions. The amount of TNF-α were calculated using the standard curve generated using recombinant proteins for TNF-α($y = 0.691*\ln(x) - 1.222$, $R^2 = 0.976$) and PGE_2 ($y = -1.10*\ln(x) + 4.426$, $R^2 = 0.995$), respectively.

2.9. Western blot analysis of iNOS and COX-2 expression

Treated RAW 264.7 macrophages were washed with iced cold DMEM and iced phosphate buffer solution; the cells were lysed with 200 µL of Laemmli buffer (BioRad) with 5% β-mercaptoethanol. The cells lysates were sonicated for 30 s and boiled for 5 min. The protein was quantified using the RCDC assay (BioRad). Western blotting was performed loading 20 µg of protein in 4-20% Tris-HCl gels (BioRad, Hercules, CA, USA) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF Hybond-P membrane (GE Healthcare, Buckinghamshire, United Kingdom), blocked, washed and subsequently incubated with either iNOS or COX-2 mouse monoclonal primary antibodies at 4°C overnight. The membranes were washed again and incubated with anti-mouse HPR-conjugated secondary antibody. The expression of the proteins was quantified using a chemiluminescent reagent (GE Healthcare, Pittsburgh, PA, USA) following the manufacturer's instructions. The membrane picture

was taken using a GL 4000 Pro Imaging system (Care stream Health, Inc., Rochester, NY, USA).

2.10. Western blot analysis of nuclear translocation of p50 and p65 sub-units of NF-κB

Separation of nuclear and cytoplasmic fractions was performed using NE-PER® nuclear and cytoplasmic reagents. RAW264.7 macrophages were seeded at a density of 2 x 10^5 cells per well and allowed to confluence for 48 h. Then, cells were treated with different concentrations of CuAqE (10, 25, 50, 75 and 100 µg/mL) and stimulated with 1µg/mL LPS for 24 h. Cells were harvested by adding 500 µL 0.25% trypsin solution. Cells were transferred to 1.5 microcentrifuge tubes and pelleted by centrifugation at 500 x*g* for 2-3 min. Extraction of nuclear and cytoplasmic fractions were performed following manufacturer's instructions with minimum modification. Briefly, cell pellet was treated with 100 µL of ice-cold cytoplasmic extraction reagent I, vortexed and incubated on ice for 10 min. Then, 5.5 µL of ice-cold cytoplasmic extraction reagent II was added and vortexed. The tube was centrifuged at 16,000 x*g* for 5 min at 4°C. The supernatant (cytoplasmic fraction) was transferred to a clean pre-chilled 1.5 microcentrifuge tube. The pellet was suspended in 50 µL nuclear extraction reagent; it was vortexed for 15 s and incubated on ice for 40 min and vortexed 15 s every 10 min. Finally, it was centrifuged at 16, 000 x *g* for 10 min at 4°C and supernatant (nuclear fraction) was collected. Samples (cytoplasmic and nuclear fractions) were stored at -80°C until use for Western blot analysis.

2.11. Multiple inflammatory proteins expression assay-antibody array membrane

RAW 264.7 macrophages were seeded at a density of 2 x 10^5 cells in six-well plates and incubating at 37°C and 5% $CO_2$for 24 h, then the medium was removed and replaced with fresh medium. Cells were treated with 50 µg/mL of the CuAqE and stimulated with 1 µg/mL of LPS and incubated for24 h. After that, cells were harvested using the kit reagent lysate and the expression of the inflammatory proteins was evaluated using the expression-antibody array membrane following the manufacturer's procedure (mouse inflammation antibody array-membrane ab133999, Abcam Inc., Cambridge, USA). The percentage of inhibition of the secretion of the proteins was calculated using the following formula: % Inhibition = 100- [(treated cells /LPS)*100].

2.12. Evaluation of reactive oxygen species (ROS)

RAW264.7 macrophages were seeded at a density of 2.5 x 10^4 cells in a 96-wells plate at 37°C and 5% CO_2, for24 h. After that, the medium was removed and replaced with fresh medium. The CuAqE (10, 25, 50, 75 and 100 µg/mL) was added and cells were stimulated with 1µg/mL of LPS. The cells were incubated at 37°C and 5% CO_2, for24 h. Then, 25 µM DCFDA was added and incubated at 37°C and 5% CO_2for6 h. Finally, florescence activity of 2' 7' –dichlorofluorescein (DCF) was quantified using a Synergy 2 fluorescent multi-well plate reader (Biotek Instruments, Winooski, Vt., USA) with excitation wavelength at 485 nm and emission wavelength at 535 nm.

2.12. Statistical analysis

Results are expressed as mean ± standard error (SEM). Data were analyzed using one way ANOVA, followed by *post hoc* Tukey's test. Membrane antibody array date was assessed by Student´s *t* test. A *p value* < 0.05 was used to determine statistical significance of data. All analyses were performed at least in three independent experiments in triplicate. In all experiments, untreated cells were considered as negative control [C(-)] and cells treated with LPS were considered as positive control [C(+)]. Statistical analysis was performed using the proc GLM procedures of SAS version 9.3 (SAS Inst. Inc., Cary, NC).

3. Results

3.1. Phenolic compounds detected by LC-ESI-MS analysis

The LC-ESI-MS analysis for phenolic compounds of the CuAqE showed a wide range of caffeoylquinic acid derivatives and glycoside flavonoids (Table 1). Representative chromatogram and mass spectra of the CuAqE by LC-ESI-MS in negative mode are shown in Fig. 1A and mass spectrometric fragmentation profile of 1 and 10 peaks (20.49 and 30.05 min) at 353.09 and 515.12 *m/z* of a caffeoyl-quinic acid and a dicaffeoyl-quinic acid respectively are shown in Fig. 1B.

3.2. Cell viability assay in RAW 264.7 macrophages

The cell viability of RAW 264.7 macrophages was not affected by CuAqE at 10, 25, 50, 75 and 100 µg/mL. The percentage of cell viability of RAW 264.7 macrophages was higher than 85 %, this value is considered to be no-toxic *in vitro* assays and results are showed in

Table 2. Therefore, the concentrations of CuAqE evaluated were considered as non-toxic in this cell line.

3.3. Effect of CuAqE on NO release and iNOs expression in LPS-stimulated RAW 264.7 macrophages

To investigate the anti-inflammatory effect of the CuAqE on LPS-stimulated RAW 264.7 macrophages, NO release and iNOS expression were evaluated. The stimulation of macrophages with LPS resulted in an increased iNOS/NO pathway signaling. This effect was significantly inhibited by the treatment with the CuAqE. The CuAqE at 25, 50, 75 and 100 µg/mL was able to decrease significantly ($p < 0.0001$) the percentage of NO release in a dose-response manner by 17.9, 57.4, 90.2 and 103.2%, respectively when compared with the positive control (Fig. 2A). Similarly, the effect of CuAqE on iNOS expression in LPS-stimulated RAW 264.7 macrophages, was also the inhibition of the expression of iNOS at 10, 25, 50, 75 and 100 µg/mL of CuAqE (64.5 ± 6.9; 40.6 ± 2.3; 33.6 ± 10.05; 28.0 ± 15.3 and 34.5 ± 18%, respectively), resulting in a strong inhibition when compared with the positive control ($p < 0.0041$) Fig. 2B.

3.4. Effect of CuAqE on COX-2 chemokines and cytokines expression in LPS-stimulated RAW 24.7 macrophages.

The treatment with the CuAqE did not cause significant changes on COX-2 expression when LPS-stimulated RAW 264.7 macrophages where treated with 10, 25, 50, 75 and 100 µg/mL or the positive control (Fig. 3). Moreover, the inhibition on COX-2 expression correlated with the suppression of PGE_2 production in LPS-stimulated RAW 264.7 macrophages (data not shown).

Table 3, shows the effect of CuAqE at 50 µg/mL on pro-inflammatory markers measured in membrane antibody array, when RAW 264.7 macrophages were stimulated with LPS. CuAqE inhibited the secretion of pro-inflammatory markers such as granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-17, IL-18, monokine induced by IFN-γ (CXCL9, MIG), stromal cell derived factor-1 (CXC12, SDF-1), monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein 1 alpha (MIP 1α), regulated on activation normal T expressed and secreted (RANTES), macrophage inflammatory protein 1 gamma (MIP 1γ, 65.2 ± 34.5%), soluble TNR receptor I (TNF RI) and soluble TNF receptor II (TNF RII). However, IL-12 p70, Interferon-inducible (CXCL11-I-TAC), thymus expressed chemokine (TECK), Lymphotactin, Keratinocyte chemoattractant (KC) and Lipopolysaccharide-induced CXC (LIX) were not-significantly different.

3.5. Effect of CuAqE on ROS production in LPS-stimulated RAW 264.7 macrophages

Intracellular levels of ROS were increased in LPS-stimulated RAW 264.7 macrophages compared to non-stimulated macrophages. This effect was strongly counteracted by the simultaneous addition of the CuAqE at 10, 25, 50, 75 and 100 µg/mL because all these concentrations inhibited significantly ($p < 0.0001$) the production of ROS when compared whit the positive control (Fig. 4).

3.6. Effect of CuAqE on nuclear translocation of NF-κB p50 and p65 subunits in LPS-stimulated RAW 264.7 macrophages

Fig. 5A and 5B shows the effect of CuAqE (10, 25, 50, 75, and 100 µg/mL) on nuclear translocation of NF-κB p50 and p65 subunits in LPS-stimulated RAW 264.7 macrophage

respectively. The expression of NF-κK p50 subunit in the nuclear fraction was significantly reduced ($p < 0.0001$) when compared with positive control. The nuclear translocation of NF-κB p65 subunit was also inhibited by all used-concentrations of CuAqE ($p < 0.0001$).

4. Discussion

In the present study we identified phenolic compounds in the CuAqE presenting a mix of caffeoyl-feruloyl-quinic acids derivatives and flavonoid glycosides. This result is in agreement with other researchers that characterized phenolic compounds in plants belonging to Asteraceae family such as *Artemisia annua* L. and *Chrysanthemum morifolium* and *Chrysanthemum coronarium* where they found a similar profile of caffeoylquinic acids derivatives and glycosyl flavones, among others compounds (Baraldi *et al*., 2008; Lai *et al*., 2007). Some of these compounds such as caffeoyl-quinic acids derivatives have been evaluated as potential anti-inflammatory, anti-oxidant, anti-parasitic, analgesic and anti-cancer agents (Upadhyay & Dixit, 2015; Puangpraphant *et al*., 2011; dos Santos *et al*., 2010; Baraldi *et al*., 2008; Han *et al*., 2008; Lai et al., 2007).

Anti-inflammatory and anti-oxidant effects of phytochemical compounds have been related with modulation of NF-κB signaling by suppressing pro-inflammatory enzymes (iNOS and COX-2), cytokines such as TNF-α, IL-6 and IL-1β, and by decreasing ROS production. In this research phytochemical compounds of CuAqE were able to reduce LPS-inflammation in RAW 264.7 by suppressing iNOS/NO pathway and ROS production. Recent studies also demonstrated that phenollic compounds of wild grape seeds (procyanidins) inhibited inflammation in the same way reducing iNOS, NO and ROS production and poncirin, a

flavonone glycoside isolated of *Poncirus trifoliate* was able to suppress nuclear translocation of NF-kB p65 and p50 sub units (Back *et al*., 2013; Kim *et al*., 2007).

It is well known that chronic low grade inflammation is involved in a range of pathophysiologic metabolic disorders and diseases such as obesity, insulin resistance, T2D, cardiovascular disease and cancer (Faloia, 2012; Balistreri *et al*., 2010; Gustafson, 2010; Tilg & Moschen, 2008; Hotamisligil, 2006). In obesity, low grade inflammation underlies with an aberrant infiltration of activated macrophages in dysfunctional adipose tissue. This systemic process involves activation of intracellular signaling pathways able to release arrested NF-κB by the inhibitor of κB (IκB) proteins, allowing its nuclear translocation, and expression of genes involved in inflammation (iNOS/NO, COX-2/PGE_2) as well as cytokines (i.e. TNF-α, IL-6, IL-1β, IL-17), chemokines (i.e. MCP-1, MIG, MIP-1α) and growth factors (GSF, MCSF). This process plays an important function in the control of apoptosis, oxidative/nitrosative stress and proliferation. Furthermore, activation of this signaling pathway is associated with persistent release of pro-inflammatory mediators, which drives to insulin resistance and chronic diseases (Xu *et al*., 2015; Tilg & Moschen, 2008; Hotamisligil, 2006). Hence, alternative phytochemical compounds targeting intracellular NF-κB signaling pathway could contribute to the anti-inflammatory process and inhibition of its associated pathologies.

In this research, LPS-stimulated conditions in RAW 264.7 macrophages promoted NF-κB nuclear translocation where it controlled transcription of pro-inflammatory genes. CuAqE was able to inhibit inflammation in LPS-stimulated RAW 264.7macrophages by suppressing the NF-κB signaling pathway, avoiding iNOS expression and finally inhibiting NO production. In the same way, preclinical-evidence showed that plant-derived phytochemicals from fruits, grains, vegetables and beverages rich in phenolic compounds

or bioactive peptides inhibited inflammation in an *in vitro* and *in vivo* models by suppressing pro-inflammatory markers (iNOS/NO, COX-2/PGE_2, TNF-α, IL-6, IL-1β) and down regulating NF-κB signaling pathways (Dia *et al*., 2014; Garcia-Diaz *et al*., 2014; Montoya *et al*., 2014; Puangpraphant *et al*., 2013; de Mejia *et al*., 2009; Martinez-Villaluenga *et al*., 2009).

Moreover, in this study, we found that CuAqE was able to decrease pro-inflammatory chemokines in LPS-stimulated RAW 264.7 macrophages such as GCSF, MCSF, IFN-γ, MCP-1, MIP 1α, MIP 1γ, RANTES, SDF-1, MIG, sTNFRI, sTNFRII and interleukins such as IL-1α, IL-6 and IL-17. Chemokines and cytokines are a family of small proteins that modulate different activities of leukocyte cells such as activation of immune cells, cell proliferation; cell trafficking, chemotaxis and recruitment of activated macrophages, T lymphocytes and neutrophils toward inflammation (Xu *et al*., 2015). In immune and metabolic organs these events propagate low grade inflammation that contributes to the development of metabolic and chronic diseases (Samaan, 2011). Taken all together, the findings of this research were that CuAqE inhibited pro-inflammatory markers associated to metabolic disorders and chronic diseases depending on low grade chronic inflammation.

On the other hand, in non-stimulated cells, ROS are generated by the normal metabolism (hydrogen peroxide, hydroxyl radical and superoxide anion radicals). The basal production of ROS the $NADP^+$/NADPH oxidase complex maintains redox homeostasis, but in overproduction, numerous detoxifying/anti-oxidative enzymatic systems are activated such as superoxide dismutase, catalase, glutathione peroxidase and hemeoxigenase-1. However, an imbalance between ROS production and inadequate anti-oxidant mechanism results in an oxidative stress state. Several researchers have reported that anti-oxidant properties of

polyphenols can produce their chemo-preventive effects through NF-κB and nuclear factor E2 related factor (Nrf2)/anti-oxidant response element (ARE) pathways activation (Devoli *et al*., 2013; Back *et al*., 2013). In this research, we found that CuAqE inhibited ROS production in a dose-dependent manner in LPS-stimulated RWA 264.7 macrophages and suggested that phenolic compounds, such as caffeoyl-quinic acids derivatives and flavonoid glycosides, could be responsible of the reduction on ROS generation by suppressing the production of NO/ROS mediating free radicals.

In summary, a possible mechanism of action by which CuAqE inhibits LPS-induced inflammation in RAW 264.7 macrophages is proposed in Fig. 6, where CuAqE is able to modulate anti-inflammatory activity through the suppression of the translocation of p50 and p65 sub-units of NF-κB that results in a reduction on iNOS expression and NO/ROS production as well as a decrease in the production of pro-inflammatory markers.

5. Conclusion

The findings of this research suggest that phenolic compounds from CuAqE, such as caffeoyl-feruloyl-quinic acids and flavonoids glygosides, might be responsible of the anti-inflammatory and anti-oxidant properties of *C. urticifolia* tea. CuAqE might produce its anti-inflammatory activity by reducing the expression of pro-inflammatory markers and by down-regulating the iNOS/NO pathway through suppression of the translocation of NF-κB. Finally, *C. urticifolia* tea could be considered as a potential beverage to prevent inflammatory disorders and their related diseases.

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Table 1. Phenolic compounds of *Calea urticifolia* lyophilized aqueous extract obtained by LC-ESI-MS analysis.

Peak number	***Compound***	***t_RT (min)***	***m/z* [M-H]$^-$**	***Chemical Formula***
1	*Caffeoyl-quinic acid*	20.59	353.087	$C_{16}H_{17}O_9^-$
2	*Caffeoyl-quinic acid*	21.61	353.087	$C_{16}H_{17}O_9^-$
3	*Caffeoyl-quinic acid derivative*	22.55	387.17	$C_{18}H_{27}O_9^-$
4	*Quinic acid derivative*	23.18	431.192	$C_{20}H_{31}O_{10}^-$
5	*Apigenin glycoside*	24.16	593.151	$C_{27}H_{29}O_{15}^-$
6	*Feruoyl-quinic acid*	25.83	367.103	$C_{17}H_{19}O_9^-$
7	*Feruoyl-quinic acid*	27.05	367.103	$C_{17}H_{19}O_9^-$
8	*Flavonoid glucoside*	27.94	425.103	$C_{20}H_{25}O_{10}^-$
9	*Caffeoyl-quinic acid derivative*	28.81	377.182	$C_{17}H_{29}O_9^-$
10	*Dicaffeoyl-quinic-acid*	30.15	515.119	$C_{25}H_{23}O_{12}^-$
11	*Dicaffeoyl-quinic-acid*	30.72	515.119	$C_{25}H_{23}O_{12}^-$
12	*Kampferol-diglycoside*	31.39	609.146	$C_{27}H_{29}O_{16}^-$
13	*Dicaffeoyl-quinic-acid*	32.74	515.119	$C_{25}H_{23}O_{12}^-$
14	*Acacetin-diglycoside*	33.04	593.151	$C_{27}H_{29}O_{15}^-$
15	*Apigenin-diglycoside*	33.73	593.151	$C_{27}H_{29}O_{15}^-$
16	*Feruoyl-quinic acid*	35.24	367.103	$C_{17}H_{19}O_9^-$
17	*Flavonoid-glycoside*	36.87	609.142	$C_{27}H_{29}O_{16}^-$
18	*Flavonoid di-glycoside*	37.39	623.161	$C_{28}H_{31}O_{16}^-$
19	*Flavonoid-glycoside*	38.50	651.16	$C_{28}H_{31}O_{17}^-$

Table 2. Effect of CuAqE on percentage of cell viability in RAW 264.7 macrophages.

Concentration	Viability (%)	*p* value
0 µg/mL	100.0 ± 11.9	
10 µg/mL	123.2 ± 11.7	$p < 0.05$
25 µg/mL	127.6 ± 24.2	$p < 0.05$
50 µg/mL	140.6 ± 14.1	$p < 0.001$
75 µg/mL	144.6 ± 11.4	$p < 0.001$
100 µg/mL	99.6 ± 15.3	$p > 0.05$

Values represent mean ± SD. $p < 0.05$ *vs* control (0 µg/mL)

Table 3. Effect of CuAqE on percentage inhibition of pro-inflammatory chemokines and cytokines in LPS-stimulated RAW 264.7 macrophages.

Inflammatory Marker	Description	Function	50 µg/mL (% inhibition)
G-CSF	Granulocyte colony stimulating factor	Cytokine induces granulocyte population from precursor cells	34.5±22.9*
M-CSF	Macrophage colony stimulating factor	Cytokine regulates production, differentiation and functions of macrophages	37.5±28.2*
IFN-γ	Interferon gamma	Macrophages activation	87.1±5.2*
IL-1α	Interleukin-1α	Pro-inflammatory cytokine induces adhesion molecules	61.6±7.3*
IL-1β	Interleukin-1β	Pro-inflammatory cytokine	29.5±28.4
IL-4	Interleukin-4	Macrophage activation, regulating cell proliferation and promotes Th2 differentiation	52.8±11.7*
IL-6	Interleukin-6	Pro-inflammatory cytokine. It plays a role in lipid metabolism and energy expenditure	88.3±8.2*
IL-9	Interleukin-9	Pro-inflammatory cytokine Stimulates proliferation of T cells, increases production of IgE	-99.2±38.1*
IL-12p70	Interleukin-12 p70	Pro-inflammatory cytokine, chemoattractant of Th1 cells and promotes IFN-γ production	30.7±21.0
IL-17	Interleukin-17	Pro-inflammatory cytokin, chemoattractant of	39.1±15.2*

		inflammatory cells and cytokines. Actives Th17 cells*	
MIG	Monokine induced by INF ϒ(CXCL9)	Involved in T cell trafficking. Chemoattractant for lymphocytes Th1	81.1 ± 4.2*
I-TAC	Interferon-inducible (CXCL11)	Recruitment of T cells to sites of inflammation	29.8±6.5
SDF-1	Stromal cell derived factor-1 (CXC12)	Chemokine that activates leukocytes and regulates trafficking of hematopoietic stem cells. Important in carcinogenesis	50.6±5.6*
MCP-1	Monocyte chemoattractant protein-1 (CCL2)	Regulates migration and infiltration of monocytes/macrophages. Recruitments macrophages in atherosclerosis	51.3±11.5*
MIP-1α	Macrophage inflammatory protein-1alpha (CCL3)	Chemokine with chemotactic activity of mononuclear leukocytes (T, B lymphocytes and monocytes)	72.2±23.6*
RANTES	Regulated on activation, normal T expressed and secreted (CCL5)	Chemoattractant of $CD8^+$, $CD4^+$ T lymphocytes, monocytes, neutrophils and dendritic cells	56.8±7.5*
MIP-1γ	Macrophage inflammatory protein-1 gamma (CCL9)	Chemokine with chemotactic activity of T cells and monocytes	65.2±34.5*
TECK	Thymus expressed chemokine (CCL25)	Chemotatic cytokine for dendritic cells, thymocytes and activated macrophages	45.2±21.0

Fractalkine	Transmembrane chemokine (CX3CL1)	Regulation of cell trafficking and chemotactic activity	-47.1±38.6*
Lymphotactin	XCL1 chemokine	Important role in cytotoxic immune response	29.8±17.7
KC	Keratinocyte chemoattractant	Recruitment of neutrophils	-18.2±10.6
LIX	Lipoplysaccharide-induced CXC chemokine	Chemokine associated with cell migration and activation of neutrophils	19.5±11.1
s TNF-RI	Soluble tumor necrosis factor receptor I	Antagonist and agonist effect of TNF-a	70.0±20.1*
s TNF-RII	Soluble tumor necrosis factor receptor II	Antagonist and agonist effect of TNF-α	76.0±13.5*

The values represent mean ± SD (n = 2 - 4). *$p < 0.05$ Student´s *t* test (LPS *vs.* treated cells).

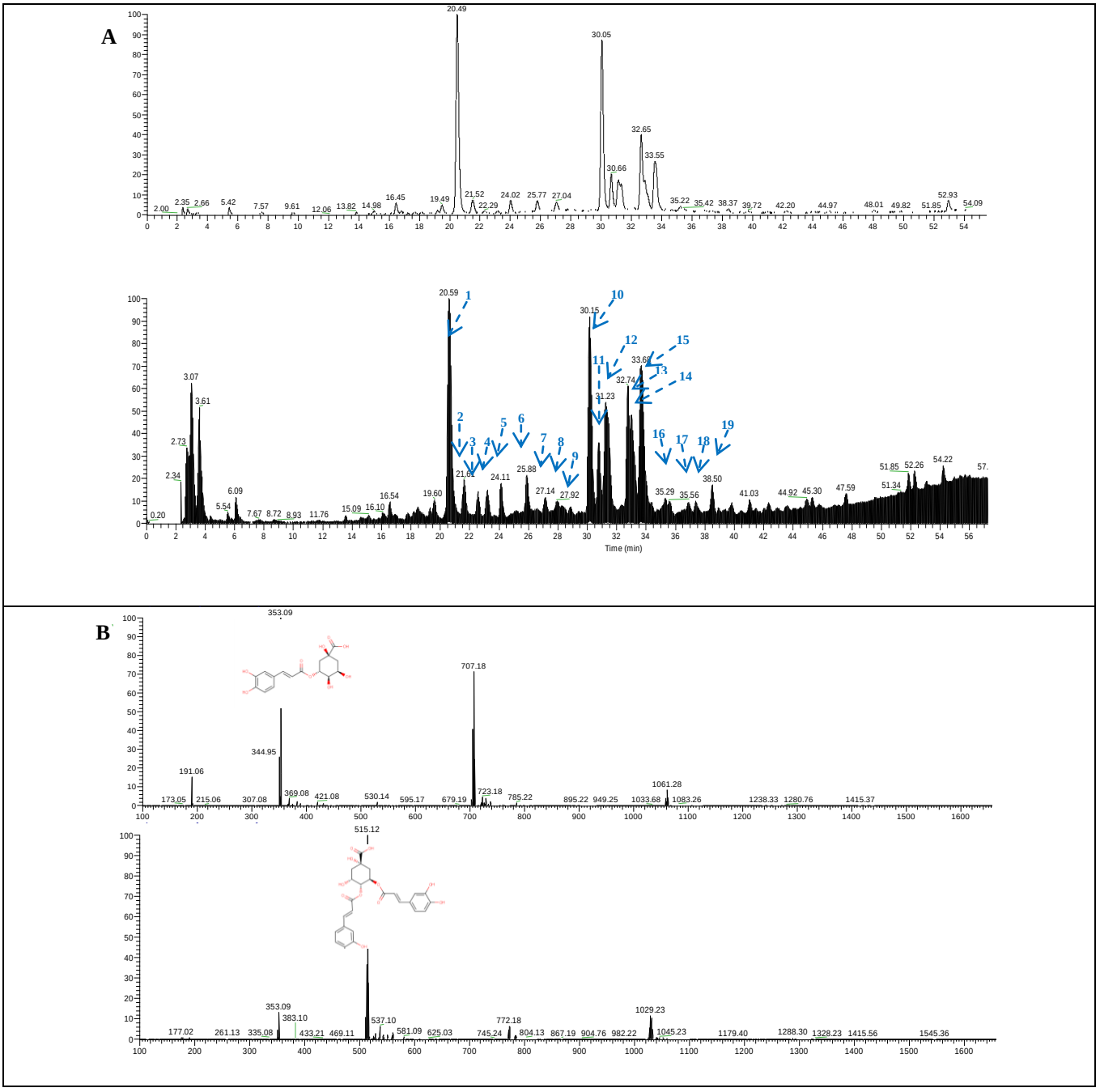

Fig. 1. **(A)** Representative chromatogram and mass spectra for phenolic compounds of CuAqE by LC-ESI-MS. **(B)** Mass spectrometric fragmentation profile of 1 and 10 peaks at 353.09 and 515.12 m/z a caffeoyl-quinic acid and a dicaffeoylquinic acid respectively.

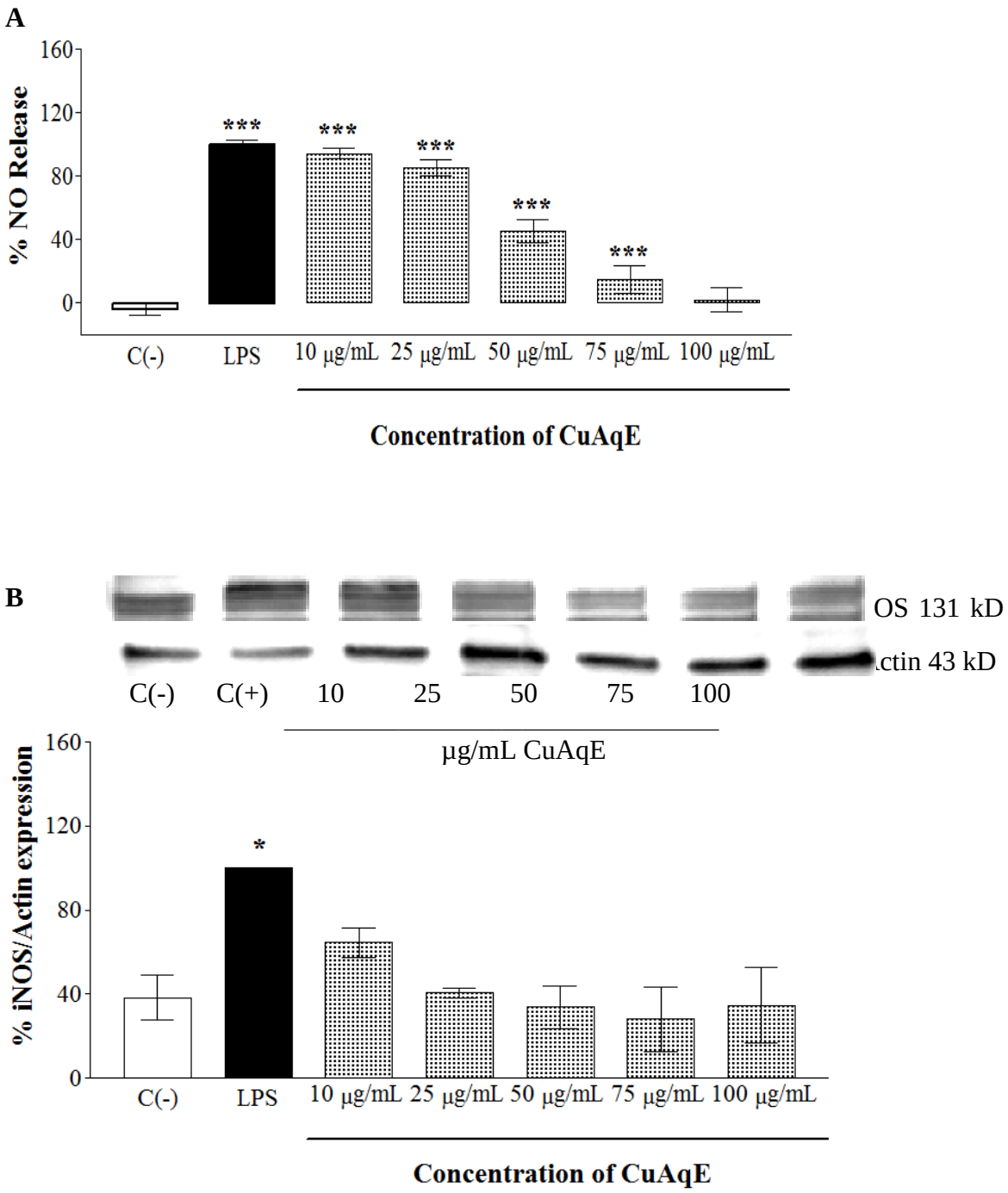

Fig. 2. Effect of CuAqE on iNOS/NO pathway modulating pro-inflammatory process in LPS-stimulated RAW 264.7 macrophages. **(A)** Presents decreased percentage of NO release (n=6) and **(B)** suppressed iNOS expression (n=3). Bars represent mean ± SEM. *p < 0.05, ** p < 0.01 and *** p < 0.001 *vs.* negative control.

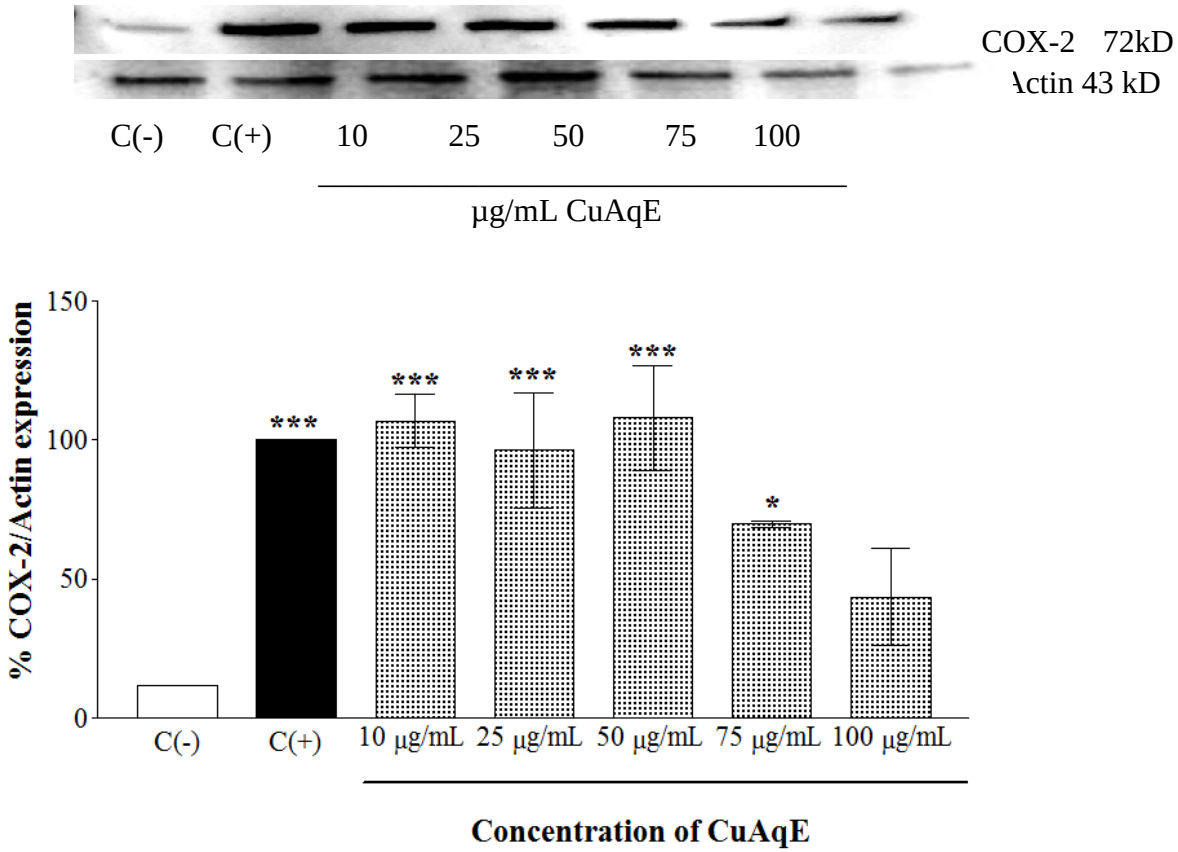

Fig.3. Effect of CuAqE on COX-2 expression in LPS-stimulated RAW 264.7 macrophages. Bars represent mean ± SEM (n=3). *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs*. negative control.

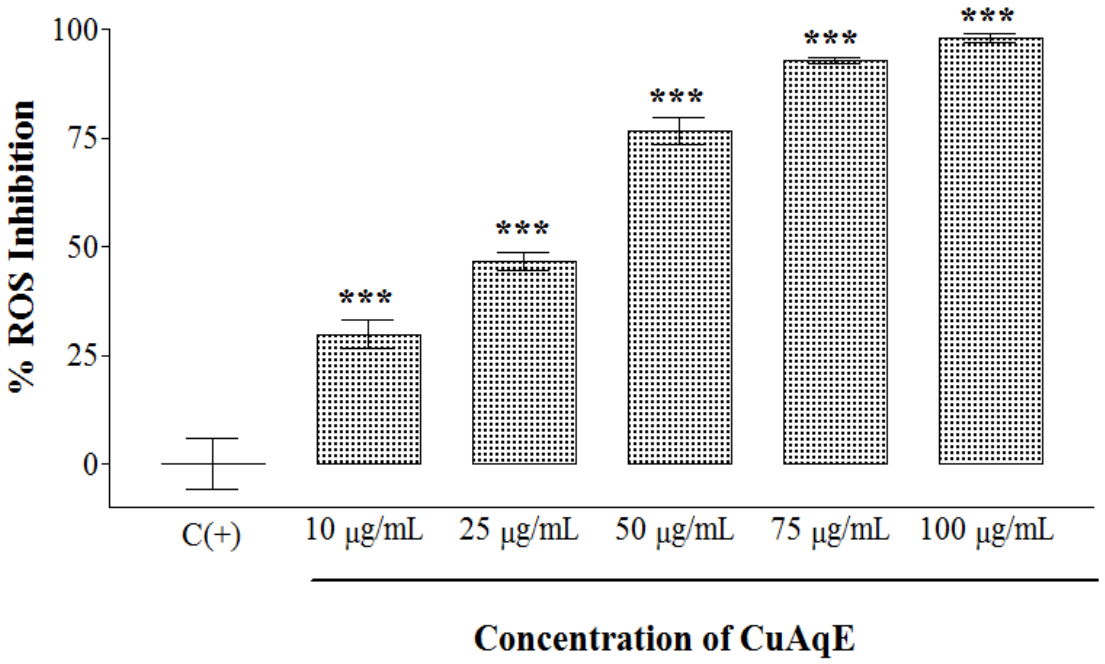

Fig. 4. Effect of CuAqE on intracellular reactive oxygen species (ROS) production in LPS-stimulated RAW 264.7 macrophages. Results showed inhibition in a dose response manner. Bars represent mean ± SEM of percentage ROS inhibition. *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs*. positive control.

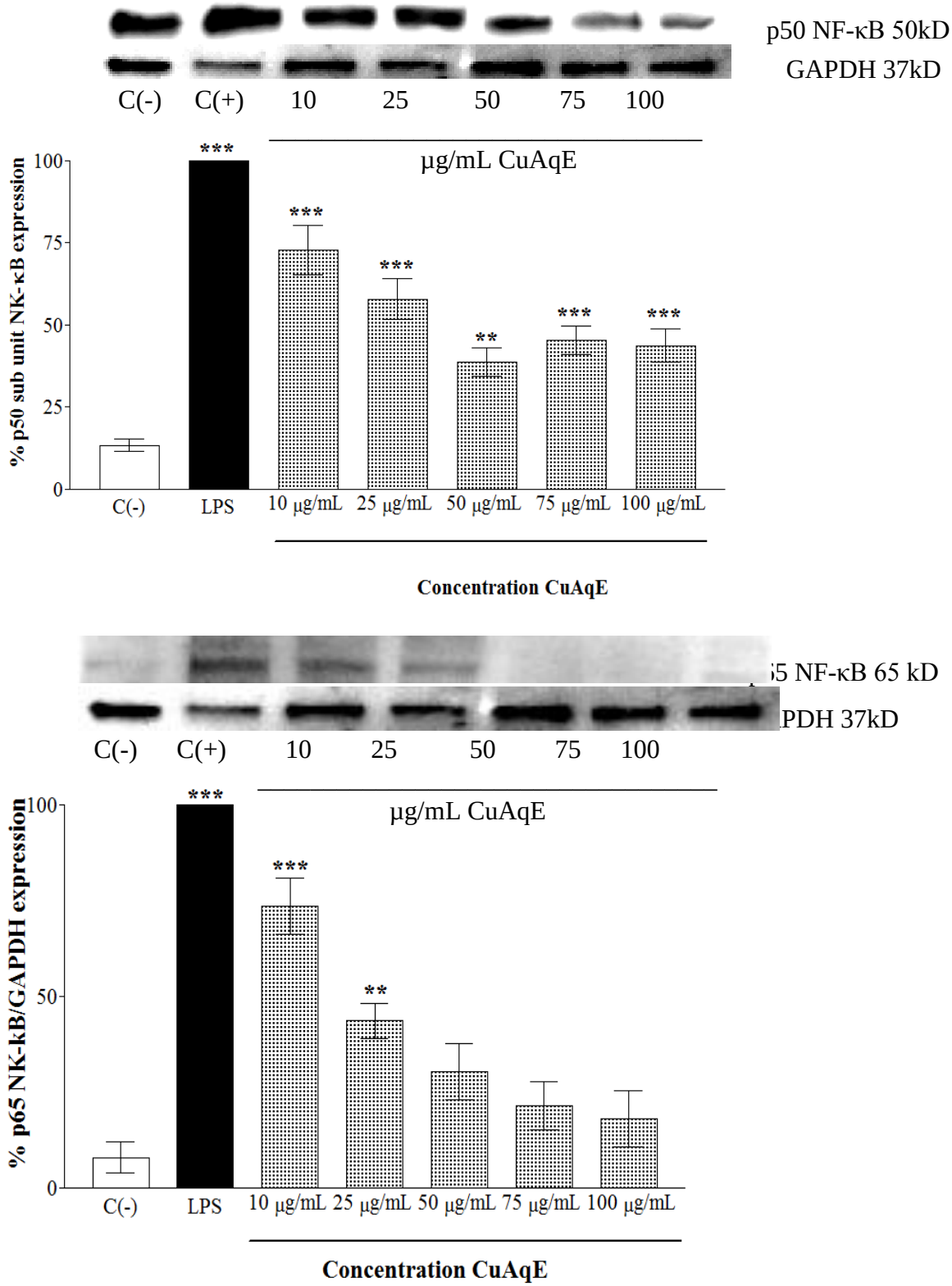

Fig. 5. Effect of CuAqE on nuclear translocation of NF-κ Bp 50 **(A)** and p 65 **(B)** sub-units in LPS-stimulated RAW 264.7 macrophages. Bars represent mean ± SEM (n=3). *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs*. negative control.

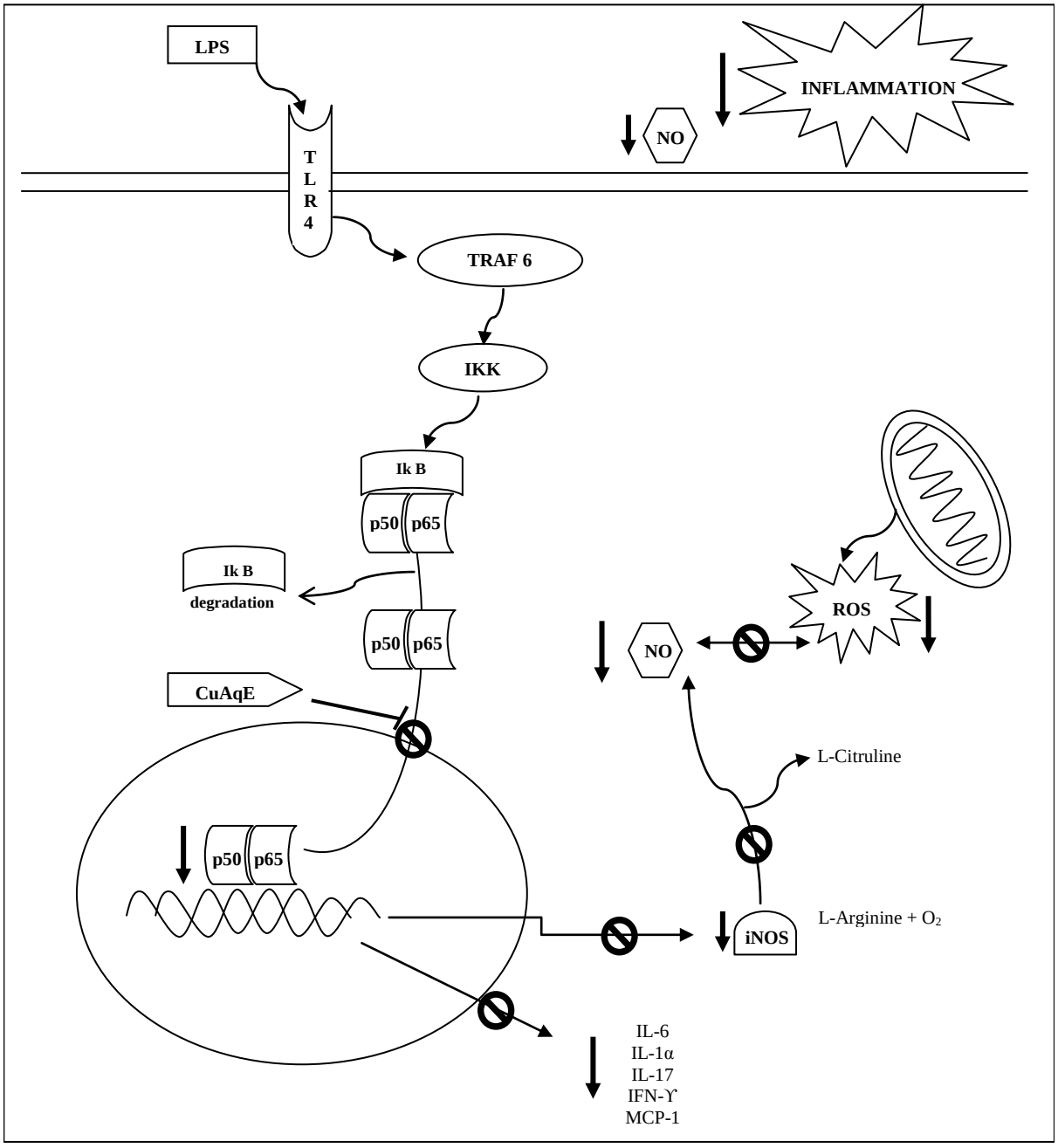

Fig. 5. Suggested mechanism of action of *Calea urticifolia* lyophilized aqueous extract (CuAqE) to inhibit LPS-induced inflammation and reactive oxygen species (ROS) production in RAW 264.7 macrophages. CuAqE inhibited nuclear translocation of nuclear factor-kappa B (NF-κB) p50 and p65 sub units resulting in suppression of expression of inducible nitric oxide synthase (iNOS) and in reduced nitric oxide (NO)/reactive oxygen species (ROS) production. LPS, lipopolysaccharide; TRAF 6, TNF receptor-associated factor 6; IKK, I kappaB kinasa; IκB, Inhibitor of κB proteins; IL, interleukine; IFN-γ, Interferon gamma; MCP-1, Monocyte chemoattractant protein-1.

III. CONCLUSIÓN GENERAL

El CuAqE no mostró efectos tóxicos *in vivo* en su evaluación aguda (DL_{50} > 5000 mg/Kg) y sub-crónica, lo que demuestra que es una sustancia segura a la dosis que se consume de manera tradicional por el grupo étnico Xi´iuy (0.055 mg/Kg), que representa 9091 veces menor que la DL_{50}.

El mecanismo de acción a nivel molecular del efecto anti-inflamatorio de CuAqE, de acuerdo con los hallazgos en esta investigación, se sugiere que está relacionado con la inhibición de los mediadores pro-inflamatorios (quimiocinas, factores quimio atrayentes, y factores de crecimiento). Con respecto al efecto anti-oxidante de CuAqE, éste se atribuye a la inhibición de iNOS/NO y ROS, que pudiera estar implicada en la modulación de la supresión de la vía de señalización del NF-κB.

Finalmente, los compuestos químicos caracterizados en el CuAqE, tales como el ácido cafeólico-quínico, así como flavonoides glicosídicos pueden ser los responsables de los efectos evaluados, ya que existen evidencias de que estos compuestos participan como anti-inflamatorios, anti-oxidantes y anti-cancerígenos.

IV. REFERENCIAS INTRODUCCIÓN GENERAL

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