

# Effects of a *Leucaena leucocephala* leaf extract on xanthine oxidase activity and serum oxypurine levels in mice

Efectos de un extracto de hojas de *Leucaena leucocephala* en la actividad de xantina oxidasa y en los niveles séricos de oxipurinas en ratones

Flavio Martínez-Morales<sup>1</sup>✉, Juan R. Zapata-Morales<sup>2</sup>✉, Juan F. López-Rodríguez<sup>3</sup>✉, Othir G. Galicia-Cruz<sup>1</sup>✉, Mario A. Isirdia-Espinoza<sup>4</sup>✉ and Othoniel H. Aragon-Martinez<sup>1\*</sup>✉

<sup>1</sup> Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de San Luis Potosí, San Luis Potosí, México.

<sup>2</sup> Departamento de Farmacia, División de Ciencias Naturales y Exactas, Universidad de Guanajuato, Guanajuato, México.

<sup>3</sup> Bioterio, Facultad de Medicina, Universidad Autónoma de San Luis Potosí, San Luis Potosí, México.

<sup>4</sup> Instituto de Investigación en Ciencias Médicas, Departamento de Clínicas, División de Ciencias Biomédicas, Centro Universitario de los Altos, Universidad de Guadalajara. Tepatitlán de Morelos, Jalisco, México.

## ABSTRACT

There is a need for novel alternatives to the medical use of allopurinol. In this sense, the present study obtained a leaf extract from *L. leucocephala*, and its chemical composition, inhibitory action against xanthine oxidase (XO) *in vitro*, inhibitory interaction between the extract and allopurinol, and the inhibitory action on XO *in vivo* using mice treated with potassium oxonate and hypoxanthine, were determined. Polyphenol and flavonoid compounds were found in the leaf extract. For the leaf extract, the IC<sub>50</sub> and maximal values were 334.60 µg/mL and 46.4 % for the inhibition of XO. The 3:1 ratio combination of allopurinol and extract showed IC<sub>50</sub> and waDRI values of 1.35 µg/mL, 1.13 (allopurinol) and 1015.72 (extract) to inhibit XO, resulting in a synergistic interaction against XO *in vitro*. This combination also enhanced the therapeutic success in the mouse model compared with allopurinol administered alone. The present study presents the first evidence for the use of an allopurinol and *L. leucocephala* extract combination at a 3:1 ratio, as a substitute for the administration of allopurinol alone.

**Keywords:** allopurinol; Chou-Talalay theory; synergism; hypoxanthine

## RESUMEN

Hay una necesidad de nuevas alternativas al uso medicinal de alopurinol. En este sentido, el presente estudio obtuvo un extracto de hojas de *L. leucocephala* al cual se le determinó su composición química, acción inhibitoria contra xantina oxidasa (XO) *in vitro*, interacción inhibitoria entre el extracto y alopurinol, y acción inhibitoria contra XO *in vivo* empleando ratones bajo administración de hipoxantina y oxonato de potasio. Compuestos polifenoles y flavonoides fueron detectados y cuantificados en el extracto de hojas. Para el extracto solo, su valor IC<sub>50</sub> y máximo de inhibición contra XO fueron de 334.60 µg/mL y 46.4 %. La combinación a una proporción 3:1 de alopurinol y extracto obtuvo valores IC<sub>50</sub> y waDRI de 1.35 µg/mL, 1.13 (allopurinol) y 1015.72 (extracto) para inhibir a XO, resultando en una interacción sinérgica contra

XO *in vitro*. Donde esta misma combinación incrementó el éxito terapéutico en el modelo murino al compararse con la administración de alopurinol solo. Nuestro estudio presenta la primera evidencia para el uso de una combinación de alopurinol y extracto de *L. leucocephala* a una proporción 3:1 como un sustituto para la administración de alopurinol solo.

**Palabras clave:** alopurinol; teoría de Chou-Talalay; sinergismo; hipoxantina

## INTRODUCTION

Uric acid (UA) is the end product of purine metabolism in humans. In the degradation pathway, xanthine oxidase (XO) transforms hypoxanthine to xanthine, which is then degraded into UA (Mehmood *et al.*, 2020). The abnormal and excess accumulation of UA in the blood, known as hyperuricemia, is a risk factor for diabetes, cardiovascular complications, metabolic syndrome, and chronic kidney disease (Chen *et al.*, 2019). Hyperuricemia can be caused by the overproduction or reduced excretion of UA (Mehmood *et al.*, 2020; Kim *et al.*, 2021a). Circulating UA is filtered by the glomeruli, and approximately 90 % of the filtered material is usually reabsorbed into the renal proximal tubules via transporter molecules (Oh *et al.*, 2019; Kim *et al.*, 2021a). As kidneys are responsible for approximately 70 % of daily UA excretion (Mehmood *et al.*, 2020), XO and renal urate transporters are important targets for the regulation of hyperuricemia in patients (Oh *et al.*, 2019).

Clinically, allopurinol is commonly used to inhibit XO activity and thus reduce serum UA levels (Chen *et al.*, 2019; Oh *et al.*, 2019). Depending on the concentration, allopurinol may cause serious side effects, such as a reaction involving eosinophilia and systemic symptoms, toxic epidermal necrolysis, Stevens-Johnson syndrome, allopurinol hypersensitivity syndrome, and gastrointestinal toxicity (Stamp and Barclay, 2018; Oh *et al.*, 2019). Consequently, there is a clear need for novel therapies for regulating serum UA levels or, alternatively, the use of allopurinol at lower doses. Plant-derived extracts have potential use as effective and natural

\*Author for correspondence: Othoniel H. Aragon-Martinez

e-mail: hugo.aragon@uaslp.mx

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antihyperuricemic therapies (Kim *et al.*, 2021a). Combination studies are common methods for identifying synergy among drugs to facilitate the reduction of doses while maintaining a similar level of therapeutic efficacy to that obtained from the use of the drug on an individual basis (Martinez-Morales *et al.*, 2022).

*Leucaena leucocephala* is a leguminous tree native to southern Mexico and northern Central America. Previous studies on the use of different parts of the tree have found antioxidant, anti-inflammatory, and anticancer properties (Chung *et al.*, 2017). To date, the effects of *L. leucocephala* extracts on *in vitro* XO activity or serum UA levels in either humans or animal models have not been evaluated, although quercetin, which is one of the major flavonoids found in the leaf extracts obtained from this plant, inhibits the generation of UA and the superoxide radicals catalyzed by XO *in vitro* (Xu *et al.*, 2018). Moreover, to our knowledge, a combination approach has never been applied to a combination of *L. leucocephala* extract and allopurinol.

The present study aimed to identify the *in vitro* synergistic inhibitory action exerted on XO, by a mixture of allopurinol and *L. leucocephala* leaf extract and, subsequently, evaluate its effects on the serum levels of UA, xanthine and hypoxanthine in an experimental model of hyperuricemia recognized in mice (Liang *et al.*, 2018).

## MATERIAL AND METHODS

### Chemicals

The 2 N Folin-Ciocalteu reagent, allopurinol, quercetin, xanthine, hypoxanthine, XO, rutin, dimethyl sulfoxide (DMSO), carboxymethylcellulose (CMC), potassium oxonate, UA, ascorbic acid, gallic acid, and creatinine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate, acetic acid, hydrochloric acid, disodium hydrogen phosphate, potassium dihydrogen phosphate, aluminum chloride, and potassium acetate were of reagent grade. Ethanol and acetonitrile were of liquid chromatography grade (Mallinckrodt Baker Inc., Mexico City, Mexico). Deionized water (MontRial, San Luis Potosi, Mexico) was used for the aqueous solutions.

### Collection and preparation of plant material

*L. leucocephala* (Lam.) de Wit samples were collected from Santa María Tonameca, Oaxaca de Juárez, Mexico, in November 2019 and authenticated by Eleazar Carranza González, who is affiliated with the Herbarium Isidro Palacios of the Instituto de Investigación de Zonas Desérticas of the Universidad Autónoma de San Luis Potosi, with a voucher specimen then deposited in the herbarium. Subsequently, the leaf samples were air-dried without exposure to the sun. Two hundred eighty-two grams of the powdered leaf sample was macerated in 1 liter of ethanol:water 96:4 v/v at room temperature (22 °C) for 12 d. The resulting solution was filtered, and the solvent was removed under reduced pressure at 40 °C. Finally, the plant extract was stored at 0 °C and protected from exposure to light.

### Identification and content of polyphenols and flavonoids in the extract

The total polyphenol content in leaf extracts was determined using a modified version of the Folin-Ciocalteu method (Musci and Yao, 2017), wherein 30 µL of leaf extract, representing 0.0112 mg dry weight, or gallic acid standard solution was mixed with 150 µL of Folin-Ciocalteu reagent diluted to 1:10 with deionized water. After 5 min at room temperature, 120 µL of a 10 % (w/v) sodium carbonate solution was added to the mixture and incubated at room temperature for 30 min. The mixture was then centrifuged at 3000 × g for 5 min at 10 °C, and 200 µL of the supernatant was placed into one well of a 96-well plate (Costar® 3595, Corning Incorporated, NY, USA), and the absorbance measured at 765 nm against a blank sample (ethanol). Polyphenol concentrations were obtained by interpolating the absorbance values of the extract samples into gallic acid calibration curves. Using the data of volume and grams of the extract used, the results are expressed as mg gallic acid equivalents/g of extract.

The total flavonoid content in extracts was quantified by a colorimetric technique (Farasat *et al.*, 2014), wherein 40 µL of leaf extract, representing 0.0150 mg dry weight, or rutin standard solution were mixed with 40 µL of a 10 % (w/v) aluminum chloride solution, 40 µL of a 1 M potassium acetate solution, and 360 µL deionized water. After 30 min at room temperature, 200 µL of the mixture was placed into one well of a 96-well plate, and the absorbance was recorded at 415 nm against blanks (ethanol). Considering the data of interpolation into rutin calibration curves and volume and grams of the extract used, the flavonoid concentration is expressed as mg rutin equivalents/g of extract.

A liquid chromatographic (LC) analysis was performed as described previously (Seal, 2016; Cefali *et al.*, 2019) for the identification of flavanols and flavones and the quantification of gallic acid, quercetin, and rutin in the extract samples. The analysis was optimized for quercetin and rutin, as these two flavonoids are described as the main compounds found in biologically relevant extracts obtained from natural sources (Cefali *et al.*, 2019). For chromatographic separation, the column compartment was maintained at 24 °C, while the mobile phase consisted of 1 % acetic acid in an acetonitrile gradient. The separation and column regeneration conditions applied were 80 % mobile phase plus 20 % acetonitrile for 2 min, followed by a change to 10 % mobile phase and 90 % acetonitrile for 8 min and a second change to 80 % mobile phase plus 20 % acetonitrile for 5 min, with the final composition then maintained for 5 min. The analysis was performed using an injection volume of 20 µL of the sample dissolved in ethanol at a 1 mL/min rate, with monitoring conducted at 257 nm, a run time of 20 min, and drawing spectra of 200 to 400 nm. Gallic acid, rutin, and quercetin were identified by means of their retention times and spectra records, while their concentration in the plant extract was obtained by interpolating their responses into calibration curves performed for each pure compound, with the results expressed as µg compound/g of leaf extract. The other flavanols and flavones

were identified using the spectral data for their B-ring cinnamoyl and A-ring benzoyl systems, which are observed as two major UV absorption peaks (Band I and Band II) in this class of compounds (Mabry *et al.*, 1970). The percentage abundance of flavanols and flavones in the extract was calculated using the area values of their peaks and all peaks eluted in the chromatogram.

### Evaluation of the inhibitory effect on XO

The assay was performed following that set out by Nguyen *et al.* (2004), with some modifications. Stock solutions of XO (0.37 U/mL) and xanthine (1 mM) were prepared in 70 mM phosphate buffer solution (pH 7.5), while stock solutions of allopurinol (1.4 mg/mL), plant extract (9.5 mg/mL), and quercetin (6 mg/mL) were prepared in ethanol:DMSO 96:4 v/v. Quercetin was used as a positive control for the action of flavonoids against XO activity. Subsequently, working standards for allopurinol, quercetin, and plant extract were prepared using ethanol:water 1:1 v/v and ranged from 0.16 to 41.30, 1.20 to 153.75, and 4.07 to 1563.00 µg/mL, respectively (seven to nine points per sample, N= 5). For each preparation tested, the number of standards and the range of concentrations were established in accordance with the guidelines to accurately estimate the concentration level that was able to achieve a 50 % inhibition ( $IC_{50}$ ) of XO activity (Sebaugh, 2011). The assay mixture comprised 100 µL of test solution, 70 µL of 70 mM phosphate buffer (pH 7.5), and 60 µL of XO stock solution. After preincubation at 37 °C for 30 min, the reaction was initiated by adding 120 µL xanthine stock solution, with the assay mixture then incubated again at 37 °C for 30 min, after which the reaction was stopped by adding 50 µL 1 N HCl. Two hundred microliters of each mixture were placed into a 96-well plate, and the absorbance was read at 290 nm.

A blank sample for each solution tested was prepared in the same way, with the enzyme solution added to the assay mixture after the addition of the 1 N HCl solution. Using the data obtained from the blank samples, the interference-free absorbance was calculated for each experimental sample. Then, the inhibitory effect on XO was expressed as the percentage of XO inhibition, which was calculated using the interference-free absorbance of the assay mixture, both with and without (0 µg/mL) the test material.

### Interaction assessment of the XO inhibitors

For the samples tested, constant ratio combinations were performed (Chou, 2006; Chou, 2010). Once the  $IC_{50}$  value for each sample was obtained via the XO inhibition assay, the combination of allopurinol and plant extract was assessed via the same assay, using six different proportions. For the combinations of allopurinol and leaf extract, concentrations ranging from 0.65 to 20.82, 0.32 to 10.37, 0.66 to 21.29, 0.24 to 7.89, 0.44 to 14.27, and 0.75 to 24.13 µg/mL were used for the 1:3, 1:1, 3:1, 5:1, 10:1, and 30:1 combination, respectively (six points per combination, N= 5). Concentrations ranging from 0.65 to 20.92, 0.32 to 10.62, 0.35 to 11.18, 0.45 to 14.61, 0.42 to 13.42, and 0.28 to 8.94 µg/mL were used for the allo-

purinol and quercetin combinations at 1:3, 1:1, 3:1, 5:1, 10:1, and 30:1, respectively (six points per combination, N= 5).

Data obtained for the combinations were evaluated using the Chou-Talalay method, which is based on the median-effect equation, which, in turn, is derived from the mass-action law principle (Chou, 2006). The Chou-Talalay theory involves the quantitative definition of synergism, additivity, and antagonism by means of a combination index (CI) value and their visual definition by means of an isobologram (Chou, 2006). Furthermore, the theory includes a dose-reduction index (DRI), which measures decreasing dose folds of each drug (Chou, 2006).

A weighted average CI (waCI) value was calculated for each combination using the following formula:  $waCI = [CI_{90} + (2 \times CI_{75}) + (3 \times CI_{50}) + (4 \times CI_{30})]/10$ . This formula was designed to increase the relevance of low effect levels, as XO inhibitors should be used at a sufficiently low concentration to achieve the target serum urate level in gout patients (Chou, 2006; Stamp and Barclay, 2018; Checkmahomed *et al.*, 2020). For synergistic or additive interactions only, a weighted average DRI (waDRI) value was determined for each component in the mixture using the formula  $waDRI = [DRI_{90} + (2 \times DRI_{75}) + (3 \times DRI_{50}) + (4 \times DRI_{30})]/10$ , with low effect levels also of high relevance for this formula.

### Animals

Once the experiments described above had been completed and their results analyzed, the utility of the leaf extract and its combined use with allopurinol in the animal model was evaluated. All animal procedures were approved by the university's Institutional Animal Care and Use Committee (approval number: BGFMUASLP-06-19) and conducted in full compliance with international guidelines (National Research Council US, 2011). Male BALB/c mice (16 - 20 g) were kept under standard laboratory conditions (room temperature  $21 \pm 1$  °C with a 12 h dark and light schedule) and housed in acrylic cages with free access to water and a standard diet. All mice were housed under laboratory conditions for one week before the experiment.

### Drug administration and animal model

Hyperuricemia was induced via potassium oxonate (uricase inhibitor) and hypoxanthine, in accordance with Lemos Lima *et al.* (2015) and Yong *et al.* (2016), with some modifications. Hypoxanthine, allopurinol, and leaf extract were suspended in 0.3 % CMC aqueous solution, while potassium oxonate was suspended in 0.5 % CMC aqueous solution. The mice were randomly divided into six experimental groups (N = 5 for each group) and subjected to fasting 2 h prior to the injection of the uricase inhibitor. The inhibitor (280 mg/kg bw, i.p.) was administered once daily to the animals from Groups 2 - 6 for three consecutive days. Vehicle (8.0 mL/kg bw), allopurinol at 2.5 mg/kg bw, leaf extract (729.2 mg/kg bw), allopurinol-extract combination at a ratio of 3:1 (2.2 mg/kg bw allopurinol plus 0.7 mg/kg bw extract), or allopurinol at 2.2 mg/kg bw were administered ten minutes after the

application of the inhibitor via gavage once a day for three days to the animals of Groups 2, 3, 4, 5, and 6, respectively.

One hour after the potassium oxonate injection, the hypoxanthine suspension was administered by gavage to the mice in Groups 2 - 6 (268.0 mg/kg bw). Group 1 was administered the vehicle via intraperitoneal and oral routes (8 mL/kg bw) for the same periods of time as the other groups. The allopurinol dose (2.5 mg/kg, Group 3) was selected to produce a uric acid level close to that found in the animals administered the vehicle (Group 1), while the doses of the extract alone (Group 4) or combined with allopurinol (Group 5) were determined on the basis of their  $IC_{50}$  values and *in vitro* interaction studies. Group 6 was administered the dose of allopurinol used in the foregoing combination but applied without extract. The oral administration of allopurinol and leaf extract was selected, as it is the most preferred route of administration in patients with hyperuricemia.

On the third day, one hour after hypoxanthine administration, mice were anesthetized with a combination of ketamine and xylazine (100 and 20 mg/kg bw, respectively), and their blood was then collected by means of cardiac puncture. Each blood sample was kept at 4 °C for 1 h and then centrifuged at 3000 × g for 10 min at 4 °C. The resultant serum samples were separated and frozen at -70 °C for further analysis.

#### Determination of serum UA, xanthine, hypoxanthine, and creatinine levels

LC analysis was performed as described previously (Tsikas *et al.*, 2004; Pleskacova *et al.*, 2017) with some modifications. For chromatographic separation, the column compartment was maintained at 32 °C, while the mobile phase consisted of a phosphate buffer solution (100 mmol/L potassium dihydrogen phosphate at pH 6.0) in an acetonitrile gradient. Separation and subsequent column regeneration conditions applied were 100 % phosphate buffer solution for 4 min, followed by a change to 70% phosphate buffer solution and 30 % acetonitrile for 30 s, with this composition then maintained for 2 min, followed by a second change to 100 % phosphate buffer solution for 30 s, with, finally, this composition then maintained for 3 min. The analysis was performed with an injection sample volume of 20 µL and a flow rate of 1 mL/min, wherein monitoring was conducted at 236, 248, 263, and 292 nm for creatinine, hypoxanthine, xanthine, and uric acid, respectively, with a run time of 10 min and drawing spectra of 200 to 400 nm. Each compound was identified by retention time and spectrum record, their concentration in the serum samples was obtained by interpolating their response into calibration curves performed with the pure compounds. The results were expressed as µmol of compound/L of serum sample.

#### Absorbance and chromatographic measurement

A Cytation™ 3 microplate reader controlled by Gen5™ software (Biotek Instruments Inc., Vermont, USA) was used for the absorbance measurements. A 1100 series Agilent LC sys-

tem consisting of a quaternary pump with degasser, a standard autosampler, a thermostatted column compartment, and a diode array detector (Agilent Technologies, Palo Alto, CA, USA) was used for the LC analyses. Agilent ChemStation software for LC 3D systems was used for data collection, integration, and evaluation of the spectra records. The chromatographic separations described above were carried out using a 150 mm long Acclaim™ 120 C18 column with a 5 µm particle size and a 4.6 mm internal diameter (Thermo Fisher Scientific, CA, USA).

#### Data analysis

For the combination analyses, the waCI value was interpreted as described previously (Chou, 2006). If the data points fell on the isobologram hypotenuse, an additive effect was determined, while if the data points fell on the lower or upper left of the hypotenuse, synergism or antagonism was determined, respectively (Chou, 2006). A waDRI of > 1 indicated a more favorable dose reduction for the combination applied than that achieved using each compound alone (Chou, 2006). Additionally, the conformity of the data with the mass-action law was evaluated using the linear correlation coefficient (r) of the median-effect plot, where an r value close to 0.9 was considered an acceptable value (Martinez-Morales *et al.*, 2022). The  $IC_{50}$  values, combination analyses, and isobolograms were conducted using Compusyn software (CompuSyn Inc, NJ, USA).

The  $IC_{50}$  values, maximal inhibitory effects (MIE), and serum oxypurine and creatinine levels are presented as the mean or mean plus standard deviation. These results were analyzed using one-way ANOVA with Tukey's posttest, which was performed using GraphPad Prism 5 software (San Diego, CA, USA). A P value of < 0.05 was considered statistically significant.

## RESULTS

### Chemical composition of the leaf extract

Table 1 shows the identification and quantification of the *L. leucocephala* leaf extract components. Rutin and quercetin comprised 7.39 % and 0.77 % of the total flavonoids, respectively, while the phenolic compound gallic acid was abundant in the leaf extract, and a nonidentified compound (Peak 1)

**Table 1.** Chemical profile of the leaf extract.

**Tabla 1.** Perfil químico del extracto de hojas.

Compound	Content in the extract
Total polyphenols	202.05 ± 21.89 mg GAE/ g extract
Total flavonoids	127.17 ± 24.62 mg RE/ g extract
Rutin	9397.4 ± 536.8 µg/ g extract
Quercetin	975.6 ± 79.5 µg/ g extract
Gallic acid	40922.0 ± 2189.6 µg/ g extract
Other flavones or flavanols	43.38 ± 0.82% of eluted peaks
Nonidentified compounds	17.60 ± 0.84% of eluted peaks

Every value includes the mean ± standard deviation (N = 5). GAE, gallic acid equivalents; RE, rutin equivalents.



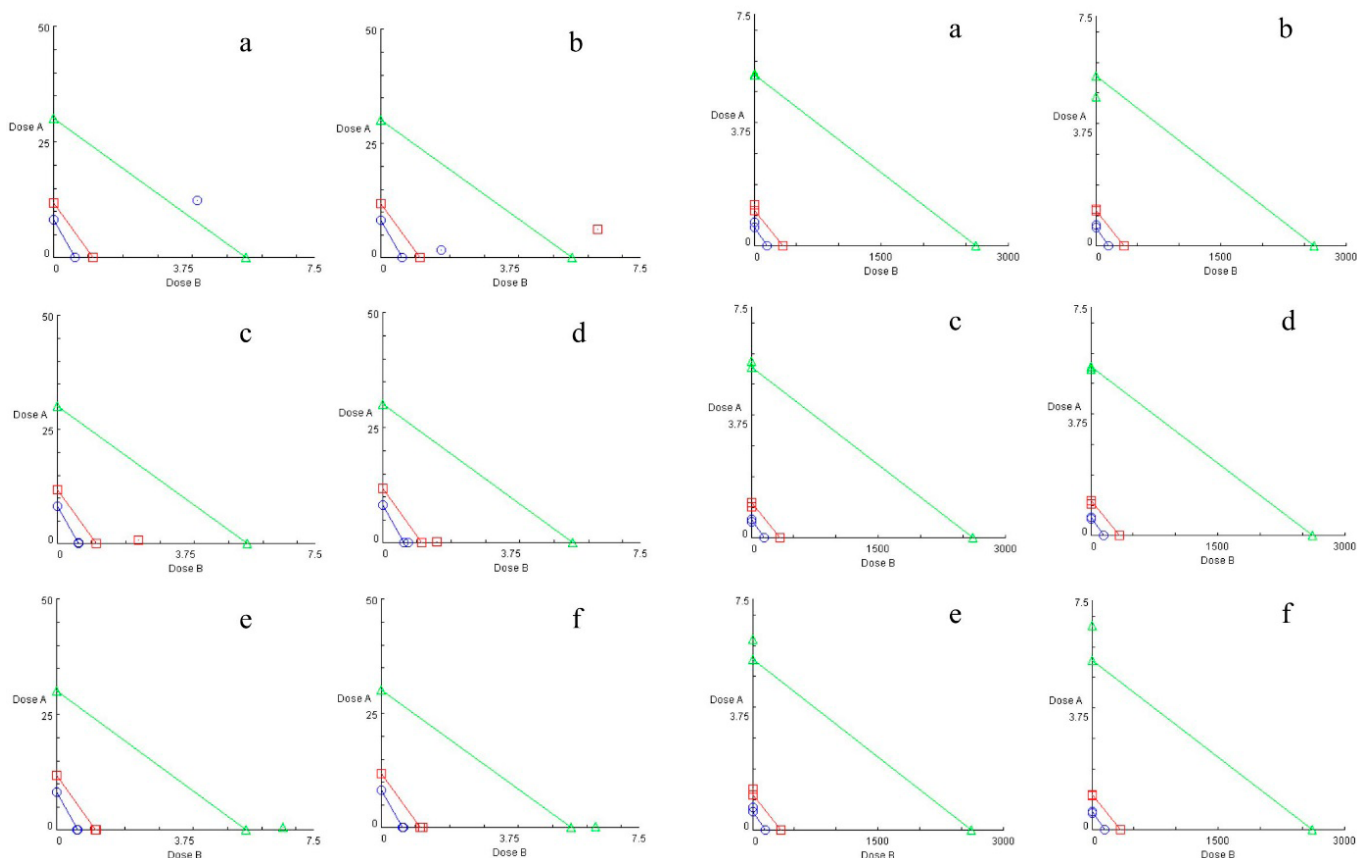
**Table 2.** Results obtained from the extract, pure compounds, and their combinations in the XO inhibition assay.**Tabla 2.** Resultados obtenidos del extracto, compuestos puros y sus combinaciones en el ensayo de la inhibición de XO.

Sample	IC <sub>50</sub> value	r value	MIE value (%)	waCI value	Type of interaction	waDRI (A / H or Q)
A	1.14	0.9661	97.8	na	na	na
H	334.60*	0.8040	46.4*	na	na	na
Q	11.80*	0.8488	40.9*	na	na	na
AH 1:3	5.42	0.9937	na	1.19	Slight antagonism	na
AH 1:1	2.39	0.9887	na	1.04	Nearly additive	0.97 / 301.69
AH 3:1	1.35	0.9609	na	0.89	Slight synergism	1.13 / 1015.72
AH 5:1	1.26	0.9836	na	0.92	Nearly additive	1.08 / 1643.50
AH 10:1	1.47	0.9538	na	1.17	Slight antagonism	na
AH 30:1	1.45	0.8958	na	1.11	Slight antagonism	na
AQ 1:3	47.35	0.8721	na	17.43	Very strong antagonism	na
AQ 1:1	12.54	0.9774	na	9.63	Strong antagonism	na
AQ 3:1	3.17	0.9809	na	3.55	Strong antagonism	na
AQ 5:1	1.90	0.9857	na	1.46	Antagonism	na
AQ 10:1	1.27	0.9905	na	1.03	Nearly additive	0.98 / 104.29
AQ 30:1	1.26	0.9900	na	1.07	Nearly additive	0.93 / 293.34

Every value includes the mean (N = 5). \*P value of < 0.0001 versus A. A, allopurinol; H, leaf extract; MIE, maximal inhibitory effect; na, not applicable; Q, quercetin.

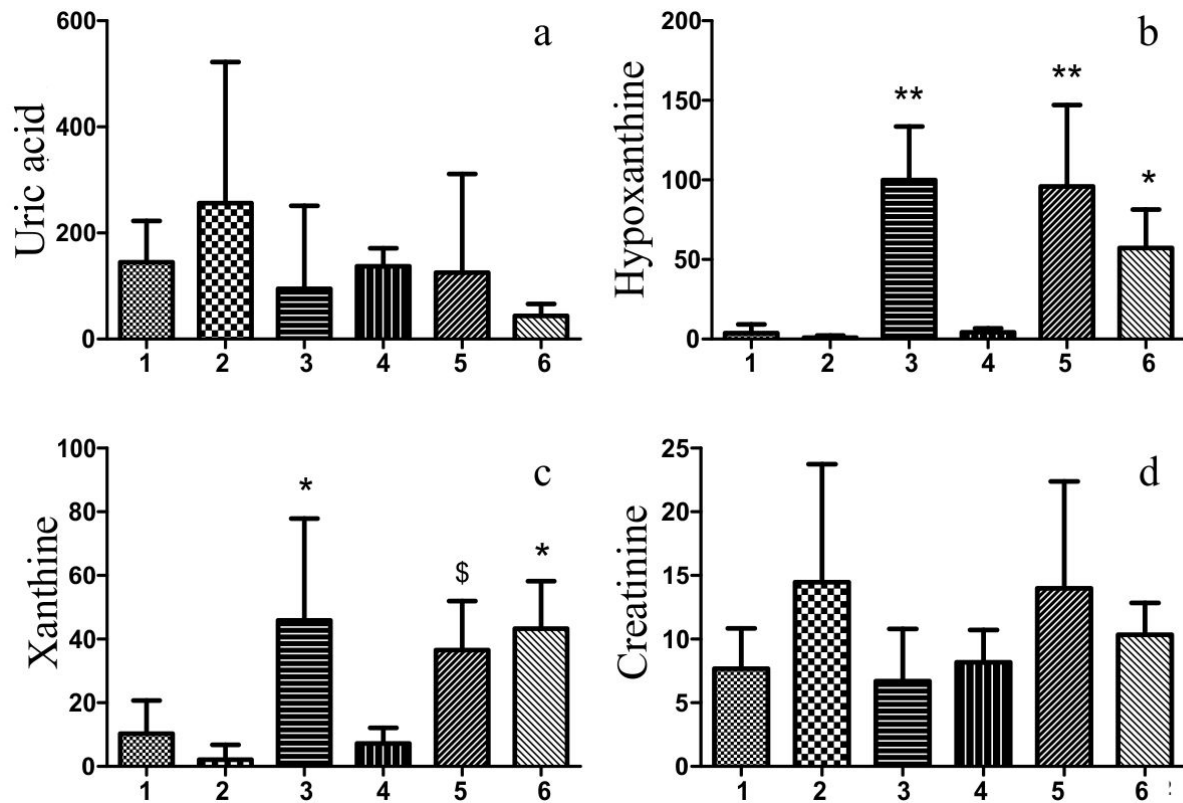
#### Assays with allopurinol and quercetin

#### Assays with allopurinol and extract



**Figure 2.** Isobolograms for the 30% (blue), 50% (red), and 90% (green) levels of XO inhibition induced by the 1:3 (a), 1:1 (b), 3:1 (c), 5:1 (d), 10:1 (e), and 30:1 (f) combinations of allopurinol and quercetin or leaf extract. For the assays with allopurinol and quercetin: A, quercetin; B, allopurinol. For the assays with allopurinol and extract: A, allopurinol; B, leaf extract.

**Figura 2.** Isobogramas de la inhibición al nivel del 30%, 50% y 90% de XO inducida por las combinaciones 1:3 (a), 1:1 (b), 3:1 (c), 5:1 (d), 10:1 (e), and 30:1 (f) de alopurinol con quercetina o extracto de hojas. Para los ensayos con alopurinol y quercetina: A, quercetina; B, alopurinol. Para los ensayos con alopurinol y extracto: A, alopurinol; B, extracto.



**Figure 3.** Serum levels ( $\mu\text{mol/L}$ ) of uric acid (a), hypoxanthine (b), xanthine (c), and creatinine (d) in mice in groups 1, 2, 3, 4, 5, and 6. \*\*P value of  $< 0.0001$  versus groups 1, 2, and 4; \*P value of  $< 0.05$  versus groups 1 and 2 in graph b. \*P value of  $< 0.05$  versus groups 1, 2, and 4; and <sup>§</sup>P value of  $< 0.05$  versus group 2 in graph c.

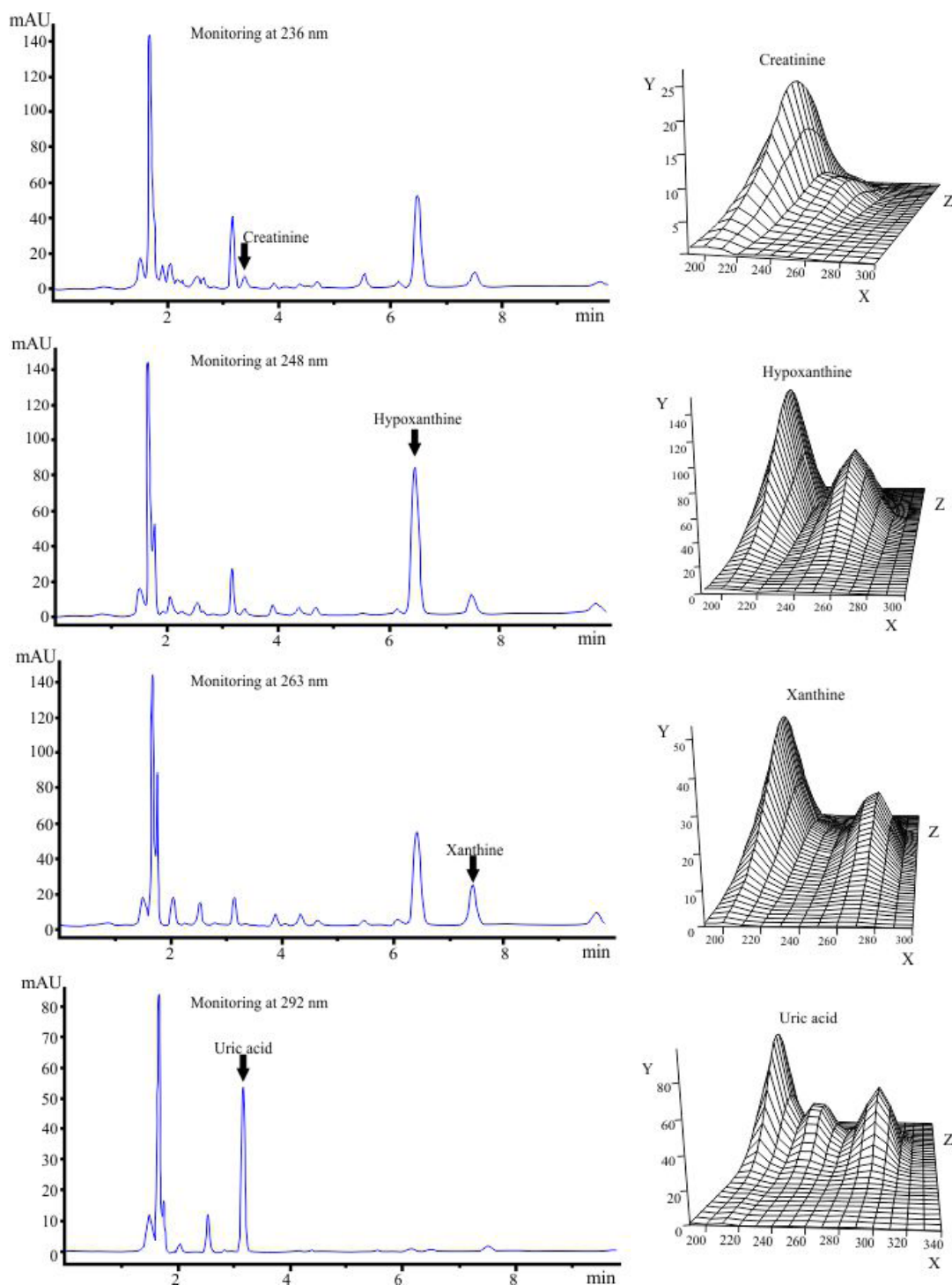
**Figura 3.** Niveles séricos ( $\mu\text{mol/L}$ ) de ácido úrico (a), hipoxantina (b), xantina (c) y creatinina (d) en los ratones de los grupos 1, 2, 3, 4, 5 y 6. \*\*Valor P de  $< 0.0001$  comparado con los grupos 1, 2 y 4; \*Valor P de  $< 0.05$  comparado con los grupos 1 y 2 en el gráfico b. \*Valor P de  $< 0.05$  comparado con los grupos 1, 2 y 4; <sup>§</sup>Valor P de  $< 0.05$  comparado con el grupo 2 en el gráfico c.

mainly flavonoids (Chew *et al.*, 2011; Xu *et al.*, 2018). Flavonoid glycosides conjugated to either arabinose and rhamnose or glucuronide, galactose, and glucose have been isolated from *L. leucocephala* leaves, with quercetin, quercetin-3-O- $\alpha$ -rhamnopyranoside, and myricetin-3-O- $\alpha$ -rhamnopyranoside described as the major flavonoids (Xu *et al.*, 2018). Other flavonoids present in low quantities include quercetin-3-O- $\alpha$ -arabinofuranose, naringenin, geraldone, 7,3'-dihydroxy-4'-methoxyflavone, apigenin, chrysoeriol, diosmetin, kaempferol, luteolin, 3',4',7-trihydroxyflavone, juglanin, kaempferol-3-O- $\alpha$ -rhamnopyranoside, (+) taxifolin, and myricetin (Xu *et al.*, 2018). Coinciding with the foregoing, analysis of the *L. leucocephala* leaf extract administered in the present study showed that it contains polyphenol compounds, a large proportion of which were flavonoids, with quercetin in low quantities, while a substantial percentage of other flavones or flavanols were also identified (Table 1 and Fig. 1).

For the first time, the present study reported the presence of gallic acid and rutin in an *L. leucocephala* leaf extract. The differences in the chemical composition and abundance of compounds in extracts obtained from the same plant species, may be due to its adaptation to the ecological environment and the extraction method used, among other

factors (Cui *et al.*, 2015; Córdova-Guerrero *et al.*, 2016). Other compounds that can be measured with the chromatographic method, such as ascorbic acid ( $\lambda_{\text{max}} = 250$  nm), catechin ( $\lambda_{\text{max}} = 279$  nm), methyl gallate ( $\lambda_{\text{max}} = 275$  nm), caffeic acid ( $\lambda_{\text{max}} = 327, 295, 243,$  and  $217$  nm), syringic acid ( $\lambda_{\text{max}} = 217$  nm), p-coumaric acid ( $\lambda_{\text{max}} = 285/305$  nm), sinapic acid ( $\lambda_{\text{max}} = 303$  or  $311$  nm), ferulic acid ( $\lambda_{\text{max}} = 285/300$  nm), myricetin ( $\lambda_{\text{max}} = 255/375$ ), apigenin ( $\lambda_{\text{max}} = 269/340$  nm), and kaempferol ( $\lambda_{\text{max}} = 268/369$  nm) were not detected in our extracts (Barthelmebs *et al.*, 2000; Galano *et al.*, 2011; Li *et al.*, 2016; Tosovic, 2017; Zhang *et al.*, 2018; Kim *et al.*, 2021b).

The *L. leucocephala* leaf extract presented a lower efficiency and potency in inhibiting the XO enzyme than allopurinol (Table 2). This finding cannot be compared with previous studies, as, to our knowledge, the present study is the first report on such effects. However, in line with our findings, the pure flavonoid rutin exhibited a higher  $\text{IC}_{50}$  value than allopurinol during *in vitro* XO inhibition assays (Malik *et al.*, 2019). *In vitro* evaluations also found that quercetin was equally or less efficient in inhibiting XO activity compared to leaf extract and allopurinol, respectively, displaying an intermediate potency compared to that observed between leaf extract and allopurinol (Table 2). The literature reports contradictory results for the *in vitro* effects of quercetin against the XO enzyme, including reports of either a greater or



**Figure 4.** Representative chromatograms obtained from a serum sample of mice pertaining to the group administered with oxonate, allopurinol, leaf extract, and hypoxanthine. Furthermore, the spectrum record for each peak is shown. The absorbance (mAU) represented on the Y-axis, the wavelength (nm) represented on the X-axis, and the time (min) represented on the Z-axis are plotted in each 3D spectrogram.

**Figura 4.** Cromatogramas representativos obtenidos de una muestra sérica de ratón perteneciente al grupo con administración de oxonato, allopurinol, extracto de hojas e hipoxantina. Además, El espectro de cada pico es mostrado. La absorbancia (mAU) en el eje Y, la longitud de onda (nm) en el eje X y el tiempo (min) en el eje Z son trazados en cada espectrograma 3D.

lesser inhibition of XO than that achieved with allopurinol (Mohos *et al.*, 2019).

To our knowledge, there are no previous reports of a combination study using allopurinol with *L. leucocephala* leaf extract and either pure rutin or quercetin. As observed in Table 2 and the isobolograms presented in Fig. 2, only the combination of allopurinol and leaf extract in a 3:1 ratio presented a slight synergistic interaction, as defined by the waCI value, which was observed at three of the four levels of effect tested (75, 50, and 30 %, with the exception of 90 %). This interaction was determined using the Chou-Talalay method, presenting an acceptable conformity with the theory applied. The synergistic interaction of the 3:1 ratio combination enabled a respective minor and major reduction of the allopurinol and extract doses required (waDRI > 1). Other allopurinol and extract combinations presented a nearly additive or slight antagonism, wherein both types of interactions did not perform better than the synergistic interaction observed, a finding supported by the IC<sub>50</sub>, waCI, and waDRI values. Consequently, the 3:1 combination of allopurinol and leaf extract was selected to be administered in mice. Moreover, the allopurinol and pure quercetin combinations did not present superior performance to, or any advantage over, the allopurinol and leaf extract combination.

The administration of potassium oxonate in BALB/c mice did not result in a significantly high level of serum UA by Day 3 (Fig. 3). This situation was produced by the different urate excretion by kidneys of the present strain rodent, compared to the mice used in the original study and by the low absorption of potassium oxonate from the peritoneal fluid into the systemic blood circulation (Bobulescu and Moe, 2012; Lemos Lima *et al.*, 2015; Wishart *et al.*, 2018; Al Shoyaib *et al.*, 2020).

The administration of an oral hypoxanthine load in animals (268 mg/kg) subjected to potassium oxonate injection, allied to the use of the LC measurement of oxypurines in serum samples (Fig. 4), enabled the adequate interpretation of XO inhibition *in vivo*. The measurement of compounds involved in the purine catabolism pathway is desirable to effectively interpret UA generation (Aragon-Martinez *et al.*, 2014). In the present study, the hypoxanthine dose administered was lower than the doses administered previously in mouse models (500 to 600 mg/kg) in studies that also applied the coadministration of potassium oxonate (Yong *et al.*, 2016; Liang *et al.*, 2018; Yong *et al.*, 2018). The administered dose was limited by the saturation of the hypoxanthine suspension and the volume of administration, as it was close to the maximum volume considered good practice in mouse models (Diehl *et al.*, 2001). We applied the XO inhibitor before the hypoxanthine load to enable the action of the inhibitor against the enzyme, which is the inverse of the administration regime applied in previous reports (Yong *et al.*, 2016; Liang *et al.*, 2018; Yong *et al.*, 2018). As observed in Fig. 3, the administration of allopurinol partially inhibited the conversion of the hypoxanthine load to xanthine, and completely inhibited the degradation of the resulting xanthine into UA in mice (Zhang *et al.*, 2021). It is important to note that there was a basal pre-

sence of serum UA in all the mouse groups tested, including the allopurinol group, as we selected an allopurinol dose that did not cause hypouricemia, a disorder associated with several inflammatory and degenerative diseases, as well as an increased risk of declining kidney function via a reduced renal antioxidant capacity (Park *et al.*, 2020).

The activity found for the combined therapy can be explained by the synergistic interaction of allopurinol with the components found in the extract, an interaction determined in our *in vitro* studies (Table 2). Moreover, the application of this interaction strengthened the evidence for the change observed in the serum hypoxanthine levels of mice, under the administration of the allopurinol and leaf extract combination compared with allopurinol alone (2.2 mg/kg) (Fig. 3). Due to the link between the P value and the Type I error, the combination of *L. leucocephala* extract and allopurinol reduced the probability of treatment failure from 28.9, which corresponds to the administration of allopurinol alone, to < 1.8 % (Gao, 2020). It is clear that the present reduction in the allopurinol dose, does not have clinical relevance, but the improved probability of therapeutic success by the use of the combination has a very important impact in real clinical settings.

Finally, the inefficacy of the *in vivo* use of the extract alone is a consequence of its limited efficiency and potency in inhibiting XO, which was observed in our *in vitro* experiments (Table 2). The present study evaluated the interactions between a drug and a natural extract, to ascertain the possibility of reducing the dose required of the drug while continuing to inhibit XO both *in vitro* and *in vivo*. We employed the Chou-Talalay method for drug combination analyses, since it has physical-chemical bearings, mathematically verifiable equations, and theories. However, there are numerous models, approaches, hypotheses, and theories that can be used instead of the current experimental design (Chou, 2006), and therefore different outcomes can be obtained. It is clear that further studies are required to obtain additional evidence to support our study, including long-term preclinical and clinical studies. The present study represents a new development for the use of synergistic combinations to inhibit XO, an important target for regulating hyperuricemia in patients.

## CONCLUSIONS

For the first time, the present study has shown that the use of an allopurinol and *L. leucocephala* leaf extract combination at a 3:1 ratio, was an effective and simple method for inhibiting XO *in vivo*, due to the synergistic interaction with an inhibitory effect on XO that is produced between the allopurinol and the compounds found in the extract. This combination produced a minor reduction in the allopurinol dose alongside an important increase in therapeutic success. Nevertheless, further studies are required to obtain data in support of the use of the allopurinol and *L. leucocephala* extract combination in a clinical setting.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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