Effects of a *Leucaena leucocephala* leaf extract on xanthine oxidase activity and serum oxyipurine 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

**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$_{50}$ 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$_{50}$ 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

**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). 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 70 % of daily UA excretion (Mehmood 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...
anti hyperuricemic 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

Leucaena 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 Potosí, 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 (Muscici 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 cinna- 
moyl 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 
flavonols 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 concentra-
tions were established in accordance with the guidelines to 
accurately estimate the concentration level that was able 
to achieve a 50 % inhibition (IC₅₀) of XO activity (Sebaugh, 2011). 
The assay mixture comprised 100 µL of test solution, 70 µL 
of 70 mM phosphate buffer solution 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 per-
centage 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 IC₅₀ 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-

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 isobolo-
gram (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 = [CI₉₀ + 
(2 × CI₇₅) + (3 × CI₅₀) + (4 × CI₃₀)]/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 = [DRI₉₀ + (2 × DRI₇₅) + 
(3 × DRI₅₀) + (4 × DRI₃₀)]/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 
der under standard laboratory conditions (room temperature 21 ± 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/
 k g 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 IC50 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 system 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 AcclaimTM 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 waCl 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 IC50 values, combination analyses, and isobolograms were conducted using Compusyn software (CombioSyn Inc, NJ, USA).

The IC50 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>
<thead>
<tr>
<th>Compound</th>
<th>Content in the extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total polyphenols</td>
<td>202.05 ± 21.89 mg GAE/g extract</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>127.17 ± 24.62 mg RE/g extract</td>
</tr>
<tr>
<td>Rutin</td>
<td>9397.4 ± 536.8 µg/g extract</td>
</tr>
<tr>
<td>Quercetin</td>
<td>975.6 ± 79.5 µg/g extract</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>40922.0 ± 2189.6 µg/g extract</td>
</tr>
<tr>
<td>Other flavones or flavanols</td>
<td>43.38 ± 0.38% of eluted peaks</td>
</tr>
<tr>
<td>Nonidentified compounds</td>
<td>17.60 ± 0.38% of eluted peaks</td>
</tr>
</tbody>
</table>

Every value includes the mean ± standard deviation (N = 5). GAE, gallic acid equivalents; RE, rutin equivalents.
coeluted with it (Fig. 1). Gallic acid, rutin, and quercetin in the extract presented wavelength absorption maxima ($\lambda_{\text{max}}$) values of 270, 260/350, and 250/370 nm, respectively (Fig. 1). Compounds (peaks) 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 were detected in extracts and exhibited $\lambda_{\text{max}}$ values at 270, 230, 270, 260/350, 260/350, 260/350, 260/350, 260/350, 260/350, 260/350, and 270/330 nm, respectively. Similar to quercetin and rutin, compounds 4 - 11 presented flavanol and flavone bands I and II. Retention times and spectra records for gallic acid, rutin, and quercetin in extracts coincided with those used as standards.

**Activity of XO inhibitors and their combinations in vitro**

Table 2 shows our IC$_{50}$, MIE, I, r, waCl and waDRI values for leaf extract and compounds, as well as the interpretation of possible interactions. Isobolograms confirmed interactions between different allopurinol combinations with either quercetin or leaf extract (Fig. 2).

**Effects of allopurinol, leaf extract, and the allopurinol and leaf extract combination in mice**

UA and creatinine serum values remained unchanged in all tested groups. Mice in Groups 3, 5, and 6 presented high hypoxanthine and xanthine serum levels, in contrast with mice in Groups 1, 2, and 4. In terms of serum hypoxanthine levels, leaf extract administration to Group 5 modified the P value in contrast to Group 6, although both groups received the same dose of allopurinol (Fig. 3).

Mouse serum chromatographic analysis revealed peaks with $\lambda_{\text{max}}$ values of 230, 250, 270, and 290 nm, which matched those of creatinine, hypoxanthine, xanthine, and uric acid standards, respectively (Fig. 4).

**DISCUSSION**

The present study shows, for the first time, that a 3:1 combination of allopurinol and *L. leucocephala* leaf extract (equivalent to 2.2: 0.7 mg/kg dose) administered to hyperuricemia-induced mice exerted a similar inhibitory effect on the XO enzyme and increased the probability of therapeutic success to that produced by allopurinol administered alone (2.5 or 2.2 mg/kg). This combination enabled significant lowering of the individual doses of both allopurinol and leaf extract required to achieve similar effects. Such effects, which were markedly noticed in the extracts, also displayed synergistic action against XO. On the other hand, leaf extract alone (729.2 mg/kg) did not achieve any inhibitory effect on XO, coinciding with observations in the *in vitro* experiments.

*L. leucocephala* leaf extracts possess a great diversity of bioactive compounds, such as tannins, vitamin E, ascorbic acid, carotenes, xanthophylls, alkaloids, and phenolics,
Table 2. Results obtained from the extract, pure compounds, and their combinations in the XO inhibition assay.

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

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. Isobologramas 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), y 30:1 (f) de allopurinol con quercetina o extracto de hojas. Para los ensayos con allopurinol y quercetina: A, quercetina; B, allopurinol. Para los ensayos con allopurinol y extracto: A, allopurinol; B, extracto.
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-α-rhamnopyranoside, and myricetin-3-O-α-rhamnopyranoside described as the major flavonoids (Xu et al., 2018). Other flavonoids present in low quantities include quercetin-3-O-α-arabinofuranose, naringenin, gerardone, 7,3′-dihydroxy-4′-methoxyflavone, apigenin, chrysoeriol, diosmetin, kaempferol, luteolin, 3′,4′,7-trihydroxyflavone, juglalin, kaempferol-3-O-α-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 flavonoids 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 (λ<sub>max</sub> = 250 nm), catechin (λ<sub>max</sub> = 279 nm), methyl gallate (λ<sub>max</sub> = 275 nm), caffeic acid (λ<sub>max</sub> = 327, 295, 243, and 217 nm), syringic acid (λ<sub>max</sub> = 217 nm), p-coumaric acid (λ<sub>max</sub> = 285/305 nm), sinapic acid (λ<sub>max</sub> = 303 or 311 nm), ferulic acid (λ<sub>max</sub> = 285/300 nm), myricetin (λ<sub>max</sub> = 255/275), apigenin (λ<sub>max</sub> = 269/340 nm), and kaempferol (λ<sub>max</sub> = 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 IC<sub>50</sub> 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, alopurinol, 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.
The synergistic interaction of the 3:1 ratio method, presenting an acceptable conformity with the 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 IC50, waCl, 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 presence 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 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.

ACKNOWLEDGMENTS
The present study was financially supported by the economic support provided to researchers by the SNI-CONAHCYT. The
authors thank Marco M. González-Chávez for their technical assistance.

CONFLICTS OF INTEREST
The authors declare that they have no conflict of interest.

REFERENCES


