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Anti-inflammatory potential of *Ricinus communis* leaves extracts *in vitro*

Ivan Luzardo-Ocampo, Ph. D. [1,2,*], M. Liceth Cuellar-Nuñez [3], Jose F. Vasco-Leal [4,*], Anayansi Escalante-Aburto [1,5]
[1] Tecnologico de Monterrey, The Institute for Obesity Research, Mexico, ivan.8907@gmail.com
[2] Tecnologico de Monterrey, School of Engineering and Sciences-Campus Guadalajara, Mexico.
[3] Advanced Biomedical Research Center, School of Medicine, Universidad Autónoma de Querétaro, liceth.cuellar@uaq.mx
[4] Posgrado en Gestión Tecnológica e Innovación, Universidad Autónoma de Querétaro, *jose.vasco@uaq.mx*
[5] Tecnologico de Monterrey, School of Engineering and Sciences-Campus Toluca, Mexico, anayansi.escalante@tec.mx
* Corresponding authors

Abstract*– Ricinus communis L. leaves are a by-product from the production of castor oil, which are commonly discarded and could be used as a source of bioactive compounds. Since most of these bioactives are polyphenols, known for their antioxidant and anti-inflammatory effects, this research aimed to characterize the polyphenolic fraction and assess the anti-inflammatory properties in vitro of R. communis leaves extracts. Two accessions of Mexican R. communis L. leaves (R1 and R2) were used. A spectrophotometric, HPLC-DAD, and GC-MS characterization of compounds from the leaves was conducted, together with their antioxidant capacity by ABTS and DPPH inhibition. An in vitro model of LPS-treated RAW 264.7 cells was used to evaluate cell viability and nitric oxide inhibition due to R1 and R2 treatment. Results indicated that R2 contained a higher amount of flavonoids, but lower antioxidant capacity than R1, suggesting that additional compounds are involved in the antioxidant capacity. (+)-catechin and rutin were the most abundant flavonoids in R2. Except for the 50 % v/v treatment, none of the R1 and R2 concentrations were cytotoxic, and inhibited nitric oxide production >60 %. The results showed that R. communis L. leaves extracts could be used to alleviate inflammation in vitro, but additional experiments regarding safety and anti-inflammatory assays are needed to fully elucidate their effect.

Keywords*—Ricinus communis, polyphenols, metabolomics, inflammation.

I. Introduction

Ricinus communis L. refers to African-origin plant species widely cultivated in Mexico due to its enhanced adaptability to particularly harsh environmental conditions, such as droughts, high temperatures, and minerals-poorly soils [1]. Although the seeds are considered the main agricultural resource of *R. communis* to produce castor bean oil and lubricants [2], among other applications, the leaves are considered to be a source of several biologically active compounds with potential health benefits that are yet to be explored [3].

The leaves have been found to be an important source of polyphenols, mainly hydroxycinnamic acids such as chlorogenic, *p*-coumaric, and caffeic acids; hydroxybenzoic acids like ellagic and gallic acids; and flavonoids, such as (+)-catechin, rutin, and quercetin [4]. Moreover, authors such as Vasco-Leal et al. [4] reported valuable amounts of oligosaccharides (raffinose, stachyose, and verbascose), antioxidant capacity measured by radicals' scavenge, and significant amounts of calcium and potassium (up to 54.99 and 30.30 ppm). However, *R. communis* leaves contain toxic components such as ricinin and ricinine, a protein and an alkaloid, respectively [5], limiting the consumption of *R. communis*-derived products [6], but their pharmacological potential, together with the polyphenolic fraction, could be used in the treatment of several conditions such as inflammation and cancer. For instance, *R. communis* compounds such as ricinoleic acid exhibit analgesic and anti-inflammatory effects, and most of the polyphenols reported for *R. communis* display anti-inflammatory effects *in vitro* and *in vivo* [7].

However, the composition of several plant species significantly changes in varied geographical locations. As *R. communis* is a widely cultivated plant in Mexico, and the leaves are considered an important by-product, this research aimed to characterize the polyphenolic composition of *R. communis* L. leaves of two representative Mexican accessions and test their anti-inflammatory properties in an *in vitro* model.

II. Materials and Methods

A. Plant material

Two different *R. communis* L. accessions were used, based on representative geographical locations at which the plant was cultivated, and named R1 and R2. R1 refers to plants grown in the state of Queretaro (Mexico) (Latitude: 20 º 43' 39.5'' North, Longitude: 100º 15' 17'' West), while R2 are Oaxacan-origin plants grown in the state of Queretaro (Mexico) (Latitude: 20 º 40' North, Longitude; 100º 27' East). The plants were harvested 131 days after the first buds appeared in winter. The plants were deposited at the "Dr. Jerzy Rzedowski" herbarium of the School of Natural Sciences of the Autonomous University of Queretaro (RCA20191 and RCA20192, respectively).

B. Polyphenolic fraction extraction, characterization, and antioxidant capacity

The free-polyphenolic fraction of the leaves was prepared based on methanolic extraction using the reported method of Vasco-Leal et al. [4]. Briefly, the leaves were dried in an oven at 90 ºC for 12 h, ground, and screened through a 250 µm sieve, and 100 mg of the powder was mixed with 10 mL HPLC-grade methanol in a 50 mL-flask and maintained under constant stirring for 24 h. The extracts were centrifuged at 3155× g in a HERMLE Z 326 K centrifuge (Hermle, Wehingen, Germany) at 4 ºC for 10 min, and then immediately protected from light

and stored at -20 ºC for further analysis. For the cell culture analysis, the solvent was eliminated using a rotatory concentrator (SpeedVac, Thermo Fisher, Waltham, MA, USA) and the resulting extracts were re-suspended in water.

For the spectrophotometric characterization of polyphenols, the Folin-Ciocalteu method [8] was used and the results were expressed as mg gallic acid equivalents/g sample [calibration curve: y=0.0904x+0.0115, R^2=0.9942], whereas the total flavonoids [9] [calibration curve: y=0.00662x+0.0978, R^2: 0.9989] and condensed tannins [10] [calibration curve: y=3.3096x+0.0101, R^2=0.9991] were reported in micrograms equivalents of rutin/g sample and milligrams equivalents of (+)-catechin/g sample, respectively.

The individual content of selected polyphenols was determined using a high-performance liquid chromatography (HPLC) analysis in an Agilent 1100 HPLC equipment (Agilent Technologies, Palo Alto, CA, USA) with a Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, 5 µm granule size) [11]. The HPLC equipment was coupled to a diode array detector (Agilent Technologies) The analysis was conducted at a 0.75 mL/min flow rate, 35 ± 0.5 ºC of column temperature, and two solvents (A: 0.1 % v/v aqueous HPLC-grade acetic acid; B: 100 % HPLC-grade acetonitrile) at an isocratic scheme as reported by Cuellar-Nuñez et al. [11]. HPLC-grade standards of gallic acid, chlorogenic acid, caffeic acid, ellagic acid, *p*-Coumaric acid, sinapic acid, (+)-catechin, rutin, and quercetin were used to elaborate standard curves to determine the concentration of each compound.

The antioxidant capacity of the polyphenolic fractions was tested by the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition as reported by Nenadis et al. [12] and Fukumoto & Mazza [13]. A Trolox calibration curve (0-800 µM) was used, and the results were expressed as antiradical activity (%) as follows:

$$\%\ ARA = \frac{Abs_{Positive\ control} - Abs_{sample}}{Abs_{Positive\ control}} \cdot 100\% \quad \text{Eq. 1.}$$

C. Metabolomic characterization of volatile compounds by Gas chromatography analysis.

A metabolomic characterization of volatile compounds from R1 and R2 accessions was conducted subjecting the samples to gas chromatography analysis coupled to mass spectrometry and solid-phase microextraction (SPME-GS/MS). The samples were analyzed and prepared as previously reported by Luzardo-Ocampo et al. [14].

D. Cell viability's test.

The anti-inflammatory effect was tested using murine RAW 264.7 macrophages [TIB-71] acquired from American Type Culture Collection [15]. The cells were cultivated under adequate conditions in a humidified 5 %-CO_2 atmosphere at 37 ºC in an incubator, using Dulbecco's modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % antibiotics, all of them acquired from Gibco (Sigma-Aldrich, St. Louis, MO, USA).

Once the cells reached 80 % confluence, the cells were transferred to 96-wells, treated with lipopolysaccharide (LPS, 1 µg/mL, Sigma-Aldrich) and 10^4 cells/well were allowed to growth for 24 h. Then, the medium was replaced by the methanolic extracts (R1 and R2; 10, 20, 30, 40, and 50 % v/v), previously prepared as indicated in section II.A., mixed with volumetric amounts of DMEM. The cells were exposed to the treatments for 24 h, the medium was eliminated and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL, dissolved in DMEM) was added, and the cells were incubated for 3 h in the incubator. Then, MTT medium was replaced by dimethyl sulfoxide (DMSO), rested for 5 min, and read in a spectrophotometer at 570 nm. Cells without treatment (untreated cells) were used as a negative control.

E. Nitric oxide test

The nitric oxide test was used to assess nitrites production in the cell culture as an indirect measurement of inflammation [16]. For this, 2.5 × 10^4 cells/well (200 µL) were growth for 24 h using 10 % supplemented-DMEM and LPS. Then, the media was replaced with R1 and R2 treatments (10, 20, 30, 40, and 50 % v/v) and the cells were incubated for 24 h. After the incubation, 100 µL of the media was added to a new 96-well plate and mixed with 100 µL of Griess Reagent prepared as indicated by the manufacturer [Griess reagent (modified), Sigma-Aldrich). The solution was incubated for 15 min, protected from light, and read at 545 nm in a spectrophotometer. A $NaNO_2$ calibration curve was used to quantify the amount of nitrites, and the inhibition was calculated based on a positive control (cells with LPS-only treatment) as follows:

$$\%\ Inh. = \frac{Abs_{Positive\ control} - Abs_{sample}}{Abs_{Positive\ control}} \cdot 100\% \quad \text{Eq. 2.}$$

G. In silico interactions

To provide further explanation about the way *R. communis'* bioactive compounds exhibit an anti-inflammatory effect, *in silico* interactions between representative polyphenols identified by HPLC-DAD and selected inflammation-associated protein markers were conducted. Hence, 3D-structures of selected polyphenols were downloaded from PubChem Database (https:// https://pubchem.ncbi.nlm.nih.gov/, accessed on May 6th, 2024): gallic acid (PubChem CID: 370), chlorogenic acid (PubChem CID: 1794427), caffeic acid (PubChem CID: 689043), ellagic

acid (PubChem CID: 5281855), *p*-coumaric acid (PubChem CID: 637542), sinapic acid (PubChem CID: 637775), (+)-catechin (PubChem CID: 9064), rutin (PubChem CID: 5280805), and quercetin (PubChem CID: 5280343). In addition, 3D structures of protein involved in the studied anti-inflammatory effect of this research article, such as inducible nitric oxide synthase (iNOS) (PDB ID: 3NQS) and the toll-like receptor 4 (TLR4, PDB ID: 3FXI), two proteins involved in the nitric oxide production in RAW 264.7 macrophages [17].

F. Statistical Analysis

The results were expressed as the mean ± S.D. of three independent experiments, in triplicates. An Analysis of Variance (ANOVA) followed by post-hoc Tukey-Kramer's test was used to assess significant differences, which were set at $p<0.05$. The analysis was conducted in JMP v. 17 software (SAS, Cary, IN, USA). The half-inhibitory concentrations (IC_{50}) of the extracts were calculated using biological equations provided by GraphPad Prism v. 8.1 software (Dotmatics, Boston, MA, USA).

III. Results

Fig. 1. Shows the spectrophotometric amount of total phenolic compounds (TPC), total flavonoids (TF), and condensed tannins (CT), and the antioxidant capacity as well of R1 and R2 extracts. As shown, R2 contained a significantly higher ($p<0.05$) amount of TF than R1, but lower antioxidant capacity.

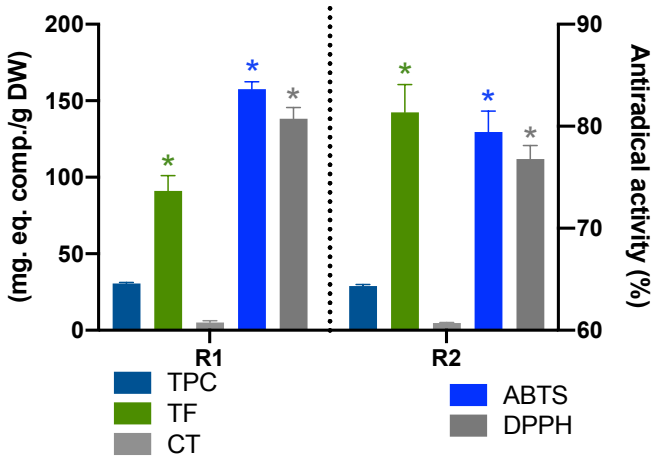

Fig. 1. Spectrophotometric content of total free-phenolic compounds (TPC, TF), and CT) and antioxidant capacity measured as antiradical activity (%) of two *R. communis* L. accessions (R1 and R2). The results are expressed as the mean ± S.D. of three independent experiments by triplicate. The asterisks indicate significant differences ($p<0.05$) between the same type of polyphenols (TPC, TF, or CT) by Tukey-Kramer's test. ABTS: 2,2'-azino-bis(3-(ethylbenzothiazoline-6-sulfonic) acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; TF: total flavonoids; TPC: total phenolic compounds; CT: condensed tannins

The identification and quantification of individual phenolic compounds by HPLC confirmed the spectrophotometric results, although p-coumaric acid was strongly abundant in R1 (Fig. 2). Of note, (+)-catechin and rutin were the most abundant flavonoids in R2, showing an overall higher amount of this polyphenolic class, compared to R1.

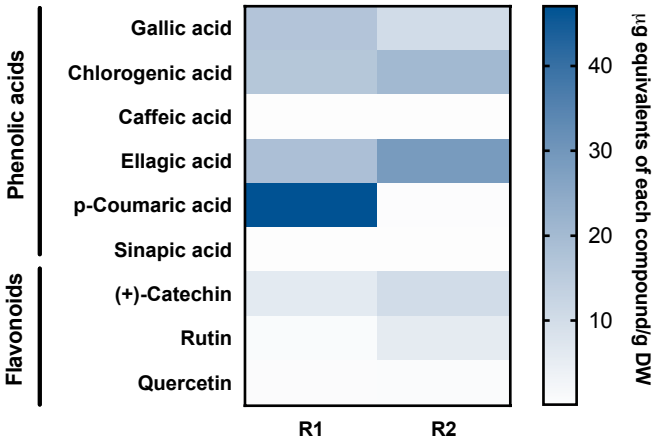

Fig. 2. Individual free phenolic compounds amount by HPLC-DAD of the two *R. communis* L. accessions (R1 and R2). The results were expressed as the average µg equivalents of each compound/g dry weight (DW).

Metabolomic characterization of volatiles showed that R2 samples contains a significantly higher amount of volatiles, mainly organic acids derivatives.

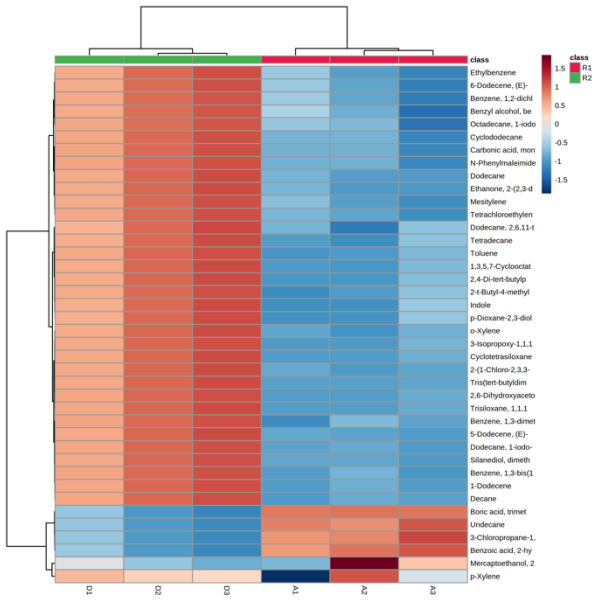

Fig. 3. Metabolomic characterization of volatile compounds from R1 and R2 accessions. The results are expressed in Log2 Abundance.

Except for the higher concentration of the extracts (50 % v/v) (Fig. 3), none of the treatments was cytotoxic as the cell viability remained ≥ 80 %. Except for the 20-50 %, the 10 % treatment stimulated cell growth by presenting viabilities > 100 %. R1 treatments preserved viability to a greater extent than R2.

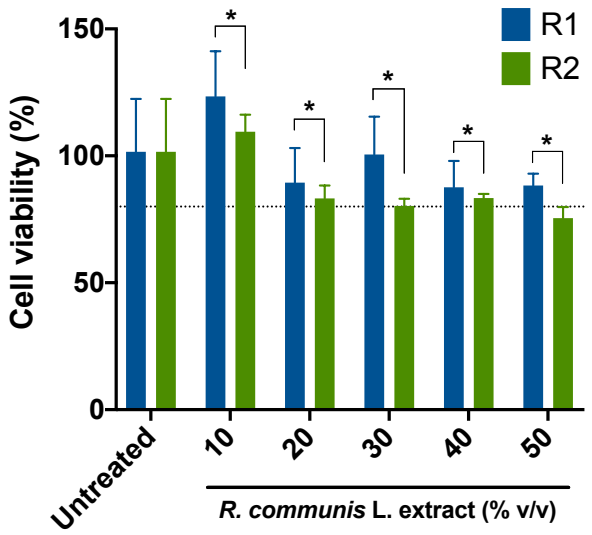

Fig. 4. Cell viability of RAW 264.7 cells against R1 and R2 accessions. The dotted line indicates 80 % viability. The results were expressed as mean ± S.D. of three independent experiments in triplicates. The asterisks indicate significant differences ($p<0.05$) between R1 and R2 for each *R. communis* L. extract by Tukey-Kramer's test.

Calculations of the IC_{50} showed similar values between R1 and R2 (Fig. 4), being R2 treatments more lethal for the cells, confirming results shown in Fig. 3. Both treatments showed a similar mathematical trend.

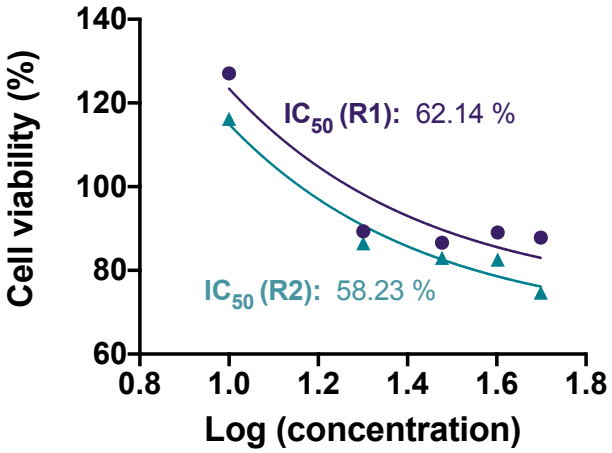

Fig. 5. Half-inhibitory (IC_{50}) calculation of the cell viability for the RAW 264.7 cells tested against R1 and R2 accessions. Calculations were performed in GraphPad Prism v. 8.1 software.

The inhibition of nitrites, indirectly indicated as nitric oxide, indicated that both treatments were effective in inhibiting this parameter in values > 60 %, but R2 was more effective in this process.

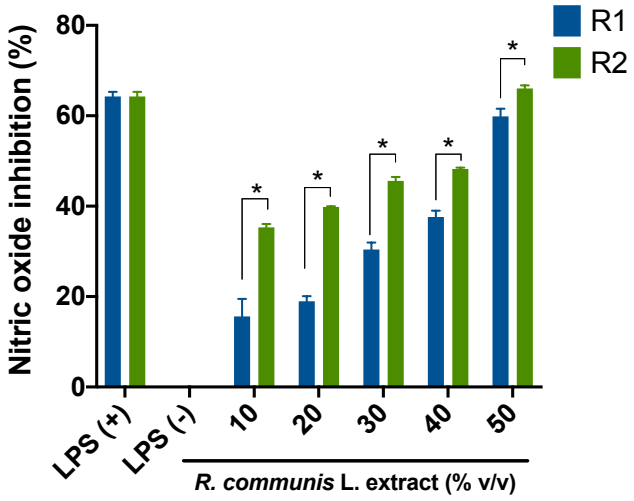

Fig. 6. Nitric oxide inhibition (%) of RAW 264.7 cells subjected to R1 and R2 treatments. The results were expressed as mean ± S.D. of three independent experiments in triplicates. The asterisks indicate significant differences ($p<0.05$) between R1 and R2 for each *R. communis* L. extract by Tukey-Kramer's test.

The potential *in silico* interactions between representative *R. communis* L. bioactive compounds and molecular targets of inflammation is presented in Fig. 7. As observed, rutin and quercetin displayed the lowest binding energies with iNOS and TLR4, suggesting the more feasible interactions. None of the other compounds reached these values (data not shown).

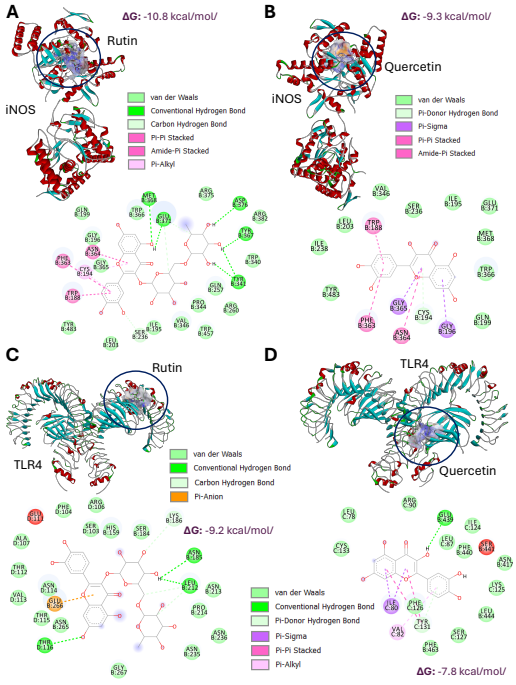

Fig. 7. *In silico* interactions between the best binding affinities of phenolic compounds identified from *R. communis* L. leaves and molecular targets of inflammation. (A) and (B) show interactions between iNOS and rutin and quercetin, respectively. (C) and (D) indicate interactions between TLR4 and rutin and quercetin, respectively.

IV. Discussion

Ricinus communis L. leaves are a valuable agricultural by-product that could be used as a source of biologically active compounds exhibiting health effects in the alleviation of chronic non-communicable diseases (NCDs) [1]. As most NCDs are based on a chronic low-inflammatory state [18], reduction of the pro-inflammatory cellular level could significantly alleviate most of these conditions. The high variety of *R. communis* plants cultivated worldwide suggests a significant amount of bioactive compounds based on the plants' metabolism, and their characterization is essential to assess the biological richness of compounds, even if the same plant is evaluated [19]. Hence, the polyphenolic characterization of Mexican-origin plants is valuable in elucidating biological properties of local plants, and the revalorization of agricultural by-products [4].

The two selected Mexican accessions evaluated in this research mostly differed in their total flavonoids, where R2 was mostly rich in (+)-catechin and rutin. It has been reported that these compounds, together with (-)-epicatechin, flavones, and kaempferol are the most abundant flavonoids in *R. communis* leaves [20]. As flavonoids were the only group of polyphenols that different between R1 and R2, it could be suggested that these compounds are not solely responsible for the reported antioxidant capacity, since R1 displayed higher ABTS and DPPH values. Hence, additional compounds, such as aromatic compounds [21] might be contributors to the antioxidant capacity, but additional research in quantifying these compounds is needed.

Untargeted metabolomic characterization has emerged as a valuable tool to elucidate the composition of bioactive compounds from samples, using well-known chromatographic procedures [22]. Our results differed slightly from a previous metabolomic characterization conducted by Vasco-Leal et al. [4], indicating secondary metabolites change due to different growing and environmental conditions. It has been reported that most of volatiles from *R. communis* leaves are involved in metabolic mechanisms such as β-alanine, glutathione, and arginine and proline metabolism, suggesting antioxidant and detoxification processes that could be valuable in targeting NCDs [21].

Inflammation is considered one of the key biological processes to reduce the effect of several NCDs, such as colorectal cancer, type 2 diabetes, and cardiovascular diseases [23]. Since several classical anti-inflammatory agents carry adverse effects [24], novel sources of anti-inflammatory and natural compounds are needed to provide an alternative to treat inflammation or at least reduce their effects [25], [26]. Most of the polyphenols found in R1 and R2 have reported antioxidant and anti-inflammatory effects *in vitro* and *in vivo* through several mechanisms such as NF-κB inhibition [27]; reduction of the expression and production of key pro-inflammatory proteins such as TNF-α, IL-6, and IL-1b ; and targeting several pro-inflammatory pathways involving NLRP3 activation [28]. The reduction of nitric oxide is an indirect measurement of inhibition of the canonical inflammation pathway [29], and both treatments were successful in this mechanism, suggesting the need for additional research exploring proteomics and nutrigenomic approaches involving the assayed samples. However, *in silico* interactions provided a research pathway where rutin and quercetin could be used as representative polyphenolic compounds from the leaves in the amelioration of specific inflammation targets such as iNOS and TLR, which are considered critical in the development of inflammation and the production of nitric oxide. Nonetheless, as *R. communis* leaves are rich in toxic compounds, a pharmacological approach might be explored, and additional experiments are needed to determine safe concentrations.

V. Conclusions

Our results suggested that *R. communis* L. exhibits a potential anti-inflammatory effect due to their content of biologically active compounds, mainly flavonoids. The assayed accessions differed on their polyphenolic composition and antioxidant capacity, indicating that additional characterization of the bioactive compounds' richness of Mexican *R. communis* leaves is needed. Additional experiments confirming the anti-inflammatory potential of the extracts and their safety are needed to provide a potential pharmacological treatment in alleviating inflammation.

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