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TCM medicinal plants showed antioxidant activity, reduced NO and TNF production, and increased IL-10 in LPS-induced RAW-264.7 cells

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Abstract

The aim of this study was to determine the in vitro antioxidant (AA) and anti-inflammatory activity of commercial plant extracts used in traditional Chinese medicine, namely: Curcubita moschata (CMO and CMV), Juniperus chinensis (JC), Peucedanum ostruthium (PO), Pinellia ternata Breit. (PTO and PTV), Rubus coreanus (RC), Rubus chingii Hu (RCH), Solanum tuberosum (STV and ST), and Viola mandshurica (VM). AA was determined by the Folin–Ciocalteu reagent reducing substances (FCRRS), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) methods. In vitro production of inflammatory cytokines in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages was determined by Griess reagent (nitric oxide [NO]) and tumor necrosis factor-α/interleukin-10 (TNF-α/IL-10) by Enzyme-Linked Immunosorbent Assay. The PTO, RCH, RC, PTV, and VM extracts had the highest FCRRS and FRAP values. RCH, RC, and VM had the highest ORAC values. All the extracts exhibited anti-inflammatory activity by reducing NO and TNF-α production or increasing IL-10. RC and JC treatments showed the highest inhibition of NO production, 68.63% and 64.91%, respectively. PTO and PTV treatments increased IL-10 production up to four-fold. The Pinellia ternata Breit. extract (PTO) was the most promising among all bioactivity assays performed, as it exhibited electron transfer-based AA activity and demonstrated anti-inflammatory effects by stimulating IL-10 production.

Keywords: antioxidants; anti-inflammatory; phenolic compounds; traditional medicines.

Practical application: Potential use in natural drugs, new supplements, and as a basis for further pharmacological studies.

1 INTRODUCTION

Inflammatory and oxidative processes are central mechanisms in the development of several chronic diseases, including obesity, diabetes, cardiovascular disorders, and cancer. These conditions are characterized by a persistent state of low-grade inflammation and increased production of reactive oxygen species (ROS), which in turn amplify tissue damage and metabolic dysfunction (Nam et al., 2015). Conventional pharmacological treatments for inflammation and oxidative stress often present high costs and undesirable side effects (Brglez Mojzer et al., 2016), which has encouraged the search for safer and more affordable alternatives.

Considering that traditional and complementary medicine plays a key role in global health, the World Health Organization (WHO, 2013) has encouraged its integration into public health systems since 2002. In Brazil, the National Policy on Integrative and Complementary Practices (PICS) includes phytotherapy among the recognized therapeutic resources (Brasil, 2006). Phyto therapeutic products, obtained from bioactive plant material (Traudi et al., 2009), often display multiple mechanisms of action due to the synergistic interaction of their phytochemical constituents, since the presence of different bioactive compounds in the same species potentiates its effects and reduces the necessary therapeutic dose (González-Muniesa et al., 2017). These molecules are known for their ability to neutralize free radicals and modulate inflammatory pathways, including the inhibition of nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activation and the regulation of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) (Brglez Mojzer et al., 2016; González-Muniesa et al., 2017; Nam et al., 2015).

Traditional Chinese medicine (TCM) represents a particularly rich source of bioactive plants, though it remains underexplored in Western countries, including Brazil. Among the numerous species used in TCM, *Curcubita moschata* (CMO and CMV), *Juniperus chinensis* (JC), *Peucedanum ostruthium* (PO), *Pinellia ternata Breit.* (PTO and PTV), *Rubus chingii Hu* (RCH), *Rubus coreanus* (RC), *Solanum tuberosum* (STV and ST), and *Viola mandshurica* (VM) stand out for their potential antioxidant (AA) and anti-inflammatory activities. However, only a limited number of studies have investigated these effects in vitro or in vivo (Joa et al., 2011; Lim et al., 2002; Medina, 2011; Yang et al., 2008), highlighting a gap in the understanding of their mechanisms and bioactive profiles.

Therefore, the present study aimed to evaluate the AA and anti-inflammatory effects of standardized extracts from these eight TCM phytotherapeutic plants in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, focusing on their capacity to modulate nitric oxide (NO), TNF, and IL-10 production, as well as their AA potential.

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1.1 Relevance of the work

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Curcubita moschata, *Juniperus chinensis*, *Peucedanum ostruthium*, *Pinellia ternata* Breit., *Rubus coreanus*, *Rubus chingii* Hu, *Solanum tuberosum*, and *Viola mandshurica* are commonly used as herbal medicine in traditional Chinese medicine. In the present study, *Pinellia ternata* Breit. extract (PTO) demonstrated the highest bioactivity potential.

2 MATERIALS AND METHODS

2.1 Materials

Folin–Ciocalteu reagent, gallic acid, [(±)-6-hydroxy-2,5,7,-8-tetramethylchromane-2-carboxylic acid] (Trolox), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), RPMI1640, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) were purchased from Sigma (Sigma-Aldrich Corporation, St Louis, MO). LPS and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). The Enzyme-Linked Immunosorbent Assay kits were purchased from BD Biosciences Pharmingen (BD Biosciences, San Diego, CA). All other chemicals and reagents were of analytical grade.

2.2 Plant extract standardization

The plant extracts of CMO and CMV, JC, PO, PTO and PTV, RC, RCH, STV and ST, and VM were obtained and donated by Florien Fitoativos (SM Empreendimentos Farmacêuticos Ltda, São Paulo, Brazil). The extracts were sourced directly from two different companies and were already standardized. Their specifications are shown in Table 1.

2.3 Sample preparation

First, 125 mg of each extract was added separately to 25 mL of distilled water for the AA experiments. Then, 500 mg of each extract was added separately to 25 mL of distilled water for the anti-inflammatory experiments. Next, it was homogenized at 9,000 × g for 30 min. The extracts were filtered, and the mixture was made up to 25 mL with distilled water. The sterilization of the extracts was performed using 0.22 μm filters (Merck Millipore, Darmstadt, Germany), and they were freeze-dried and stored at −20°C until their use.

2.4 In vitro antioxidant assays

2.4.1 Folin–Ciocalteu reagent reducing substances (

The procedure was carried out according to Medina (2011). First, 450 μL of distilled water and 50 μL of extract, gallic acid standard solutions (50, 100, 200, 300, 400, 500, 600 μg/mL), or distilled water for the blank were added and mixed. The Folin–Ciocalteu reagent (50 μL) was added and mixed. Then, 500 μL of 7% Na_2CO_3 and 200 μL of distilled water were added and mixed. The mixture was left to react at room temperature in the dark for 90 min. The absorbance was measured at 765 nm, and the results were expressed as mg gallic acid equivalent (GAE) per gram of sample (mg GAE/g sample).

2.4.2 Ferric Reducing Antioxidant Power

First, 2.5 mL of 20 mM ferric chloride solution ($FeCl_3$), 2.5 mL of 10 mM TPTZ solution, and 25 mL of 0.3 M acetate buffer were added for each 30 mL of FRAP reagent. Then, 30 μL of extract, standard (100, 200, 400, 800, 1,200, and 1,600 μM), or blank was mixed in the dark with 90 μL of water and 900 μL of FRAP reagent. The mixture was incubated at 37 °C for 30 min. The absorbance was measured

Table 1. Identification of the plant extracts*.

Scientific name	Abbreviation	Extract manufacturer	Standardization
Curcubita moschata	CMO	Organic Herb	10:1
	CMV	VNI	10:1
Juniperus chinensis	JC	Organic Herb	Quercetin 40%
Peucedanum ostruthium	PO	VNI	Saponins 5%
Pinellia ternata Breit.	PTO	Organic Herb	Tannins 15%
	PTV	VNI	Tannins 5%
Rubus coreanus	RC	VNI	Ellagic acid 5%
Rubus chingii Hu	RCH	VNI	Ellagic acid 5%
Solanum tuberosum	STV	VNI	Flavonoids 5%;
	ST	VNI	Saponins 5% Flavonoids 5%
Viola mandshurica	VM	VNI	Saponins 5%

*The extracts were obtained from two companies: VNI – Qingdao Vital Nutraceutical Ingredients BioScience Co. (Shandong Province, P.R. China) and Organic Herb – Changsha Organic Herb Inc. (Changsha, P.R. China). Information regarding the standardization of the extracts was provided by the respective companies.

at 595 nm, and the results were expressed as mg Trolox equivalent (TE) per gram of sample (mg TE/g sample) (Dávalos et al., 2004).

2.4.3 Oxygen radical absorbance capacity

The ORAC assay was performed according to Dávalos et al. (2004). We initially used 20 μl of the extract or standard (Trolox 25, 50, 100, 300, 500, and 700 μM), 120 μL of sodium fluorescein in potassium phosphate buffer (pH 7.4) (final concentration 0.378 μg/mL), and 60 μl of AAPH (108 mg/mL). Fluorescence was measured every minute for 80 min with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The AA capacity was expressed as μmol TE per gram of sample (μmol TE/g sample), based on the area under the curve (AUC) for the decline in the fluorescence time.

2.5 In vitro cell-based assays

2.5.1 Cell culture and treatments

A RAW 264.7 murine macrophage cell line was used in this study (kindly provided by Juliana Alves Macedo, UNICAMP, Brazil) to conduct the viability assay and to assess the production of inflammatory mediators. The cell line was cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C in 5% CO_2.

2.5.2 Cell viability assay

The cell viability assay was assessed by MTT method (Silva, 2016). Cells were seeded into 96-well microplates at a density of 1×10^5 cells/well and incubated for 24 h. After removing the supernatants, cells were incubated with extracts (2, 1, 0.5, 0.25, and 0.125 mg/mL) in complete RPMI medium and 10 μL of 5 mg/mL MTT in pH 7.2 phosphate-buffered saline (PBS) for 4 h at 37 °C. The supernatants were removed, and formazan crystals formed by MTT reduction in living cells were dissolved in dimethylsulfoxide (DMSO). The optical density was measured at 540 nm. The results were expressed as relative cell viability (%) using the blank treatment (RPMI 1640 complete medium) as control.

2.5.3 Production of inflammatory mediators

Raw 264.7 cells were seeded into a 24-well culture plate at a density of 4×10^5 cells/well and incubated for 24 h at 37 °C. After removing the supernatants, cells were treated with extracts (final concentration: 1.5, 0.75, and 0.375 mg/mL), inflammatory stimulus (1 μg/mL LPS), and extracts combined with inflammatory stimulus for 24 h or blank (RPMI 1640 complete medium). Two independent experiments were performed in duplicate. The supernatants were removed and stored at −80°C for further determination of inflammatory markers.

2.5.4 Determination of nitrite production

NO released into the supernatants of the cell cultures was indirectly determined using a quantitative colorimetric assay based on the Griess reaction (1% sulphanilamide, 2.5% phosphoric acid, and 0.1% naphthylethylenediamine). Next, 50 μL of supernatant was mixed with 50 μL of Griess reagent in a 96-well microplate. The optical density was then measured at 540 nm in a microplate reader, and the concentration of nitrite in the samples was determined by comparison with a standard curve of sodium nitrite (250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, and 0 μM). Untreated and LPS-stimulated cells were used as the blank control. Two independent experiments were performed in duplicate.

2.5.5 Determination of tumor necrosis factor-alpha and interleukin-10

The TNF-α and IL-10 levels were measured in macrophage supernatants according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA). Absorbance was read at 450 nm in a microplate reader. Untreated and LPS-stimulated cells were used as the blank control.

2.6 Statistical analysis

The statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA). The dataset was evaluated for normal distribution.by D'Agostino & Pearson. The statistical significance of differences was determined by one-way analysis of variance (ANOVA), followed by Tukey's test for AA assays and Bonferroni's test for comparison between control and treatment groups in anti-inflammatory assays. The results were expressed as mean ± standard deviation. Values were considered significant at $P < .05$.

3 RESULTS AND DISCUSSION

3.1 Evaluation of in vitro antioxidant assays

The FCRRS is a non-specific method for phenolic compounds (Kupina et al., 2018; Magalhães et al., 2008). The extracts with the highest FCRRS (Figure 1A) were PTO (97.90 mg GAE/g sample), followed by RCH (83.03 mg GAE/g sample). The PTO showed 45.17% higher total reducing capacity than PTV, which can be explained by the higher quantification of tannins in the first extract when compared to the second. The RCH extract showed a 38.15% higher total reducing capacity than the RC. Both are standardized with 5% ellagic acid, commonly found in these species (Shahidi & Ambigaipalan, 2015). The difference in the reducing capacity of the samples may be due to the action of other phenolic compounds in the extracts, which preferentially act as AAs by electron transference. This suggestion also comes from the chromatographic profile of both, indicating the presence of other compounds containing aromatic rings (data not shown).

The VM extract showed the third-highest FCRRS value. Possibly, the saponin content was responsible for this reducing capacity (Biswas, 2016), since the FCRRS method is not specific for phenolic compounds. Furthermore, the chromatogram of the sample also indicates the presence of compounds with aromatic rings in their structure, which are known to react with the Folin–Ciocalteu reagent (Figure S1).

The extracts with the highest AA capacities by the FRAP method (Figure 1B) are the same ones with the highest contents of total reducing substances in the Folin–Ciocalteau reagent (Figure 1A), both based on electron transfer.

The PTO showed the highest AA activity by the FRAP method (482.18 μM TE/g sample). In turn, the RC (311.25 μM TE/g sample), RCH (307.10 μM TE/g sample), PTV (293.17 μM TE/g sample), and VM (260.20 μM TE/g sample) extracts showed the second-highest AA activities by this method.

The tannins present in the PTO and PTV seem to act as AAs by electron transfer, since the AA activity of both was well performed in methods of this type. Studies assessing these species have observed AA activity, although most investigations focused on isolated compounds rather than crude extracts (Liu et al., 2017; Wu et al., 2015).

The FRAP and ORAC AA methods can be used in combination to distinguish the mechanisms of action of a given sample (Shahidi & Zhong, 2015). FRAP is a non-competitive, non-radical method that measures the ability of the AA to reduce the ferric ions to ferrous ions by electron transference. In turn, the ORAC is a competitive method in which the AA competes with the peroxyl radical for the substrate, donating a hydrogen ion for its neutralization, simulating physiological conditions. Thus, ORAC measures the ability of the AA to break the radical chain.

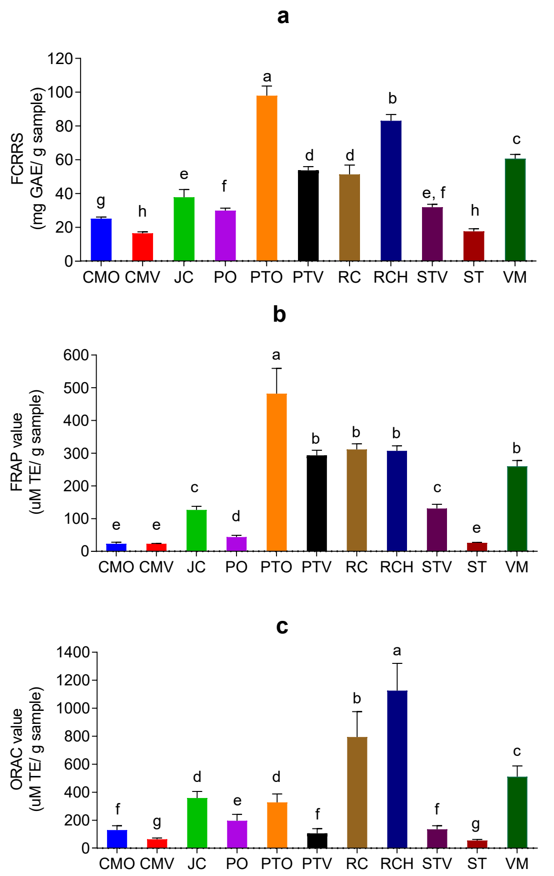
The RCH (1,126.41 μM TE/g sample), RC (794.68 μM TE/g sample), and VM (511.27 μM TE/g sample) extracts showed the highest ORAC values (Figure 1C). These former plant extracts are also among the highest values in the previous methods, which indicates action by both mechanisms. The possible presence of different compounds (chromatographic peaks — Supplementary Material) suggests that RCH, RC, and VM extracts have different phenolic compounds with distinct AA predominant mechanisms.

Given that it also exhibited the highest values in previous assays, the RCH extract, together with PTO, was identified as having the strongest AA activity based on the methodologies employed.

3.2 Evaluation of in vitro cell-based assays

The plant extracts were evaluated for cytotoxicity at concentrations of 0.125, 0.25, 0.5, 1, and 2 mg/mL (Supplementary Material). The extracts had no cytotoxic effects on RAW 264.7 cells, so these concentrations served as the basis for the subsequent cellular assays, in which the final concentrations in the wells were 1.5, 0.75, and 0.375 mg/mL.

Then, the effects of the extracts on NO and cytokine production were satisfactorily induced by LPS in RAW 264.7 cells to evaluate the anti-inflammatory activity (Figures 2 and 3). LPS is an endotoxin found in the membrane of Gram-negative bacteria, capable of stimulating Toll-like receptor (TLR-4), which promotes NF-κB activation, resulting in increased production of pro-inflammatory cytokines (Marimoutou et al., 2015). Stimulated by LPS, NF-κB translocates to the macrophage nucleus and activates the transcription of several genes involved in the inflammatory response, such as inducible nitric oxide synthase (iNOS), responsible for NO production, and inflammatory cytokines, such as TNF-α (Jais & Brüning, 2017; Manna & Jain, 2015). The anti-inflammatory activity occurs through the inhibition of pro-inflammatory cytokines, indirectly by reducing ROS levels that activate NF-κB, or by stimulating the production of anti-inflammatory cytokines (Yahfoufi et al., 2018).



CMO and CMV: *Curcubita moschata*; JC: *Juniperus chinensis*; PO: *Peucedanum ostruthium*; PTO and PTV: *Pinellia ternata Breit.*; RC: *Rubus coreanus*; RCH: *Rubus chingii Hu*; STV and ST: *Solanum tuberosum*; VM: *Viola mandshurica*.

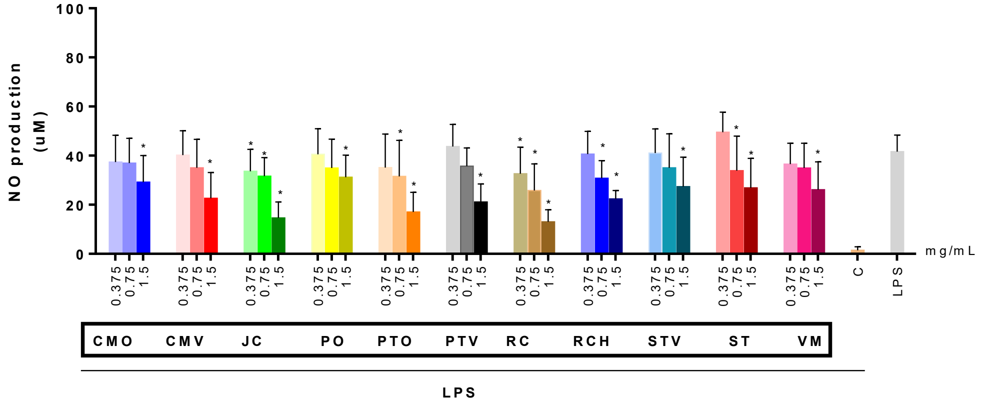
Figure 1. Folin–Ciocalteu reagent reducing substances (A), FRAP (B), and ORAC (C). The difference between the extracts was evaluated by one-way ANOVA, followed by Tukey's post hoc test. The results are represented by the mean±standard deviation. The column of the extract represented by the letter "a" presents the highest value of the experiment. The subsequent letters represent a descending order of values (a–h). Columns with different letters from each other represent differences between the extracts represented. The X-axis indicates the acronym of the aqueous extracts; the Y-axis indicates the values obtained in the experiment.

3.2.1 Production of nitric oxide in cells treated with herbal medicine

All plant extracts showed an inhibitory effect on NO with a reduction in its production. The RC and JC extracts were able to reduce the capacity of NO production by cells up to 68%. Moreover, they promoted inhibition of NO in all concentrations tested in a dose-dependent manner.

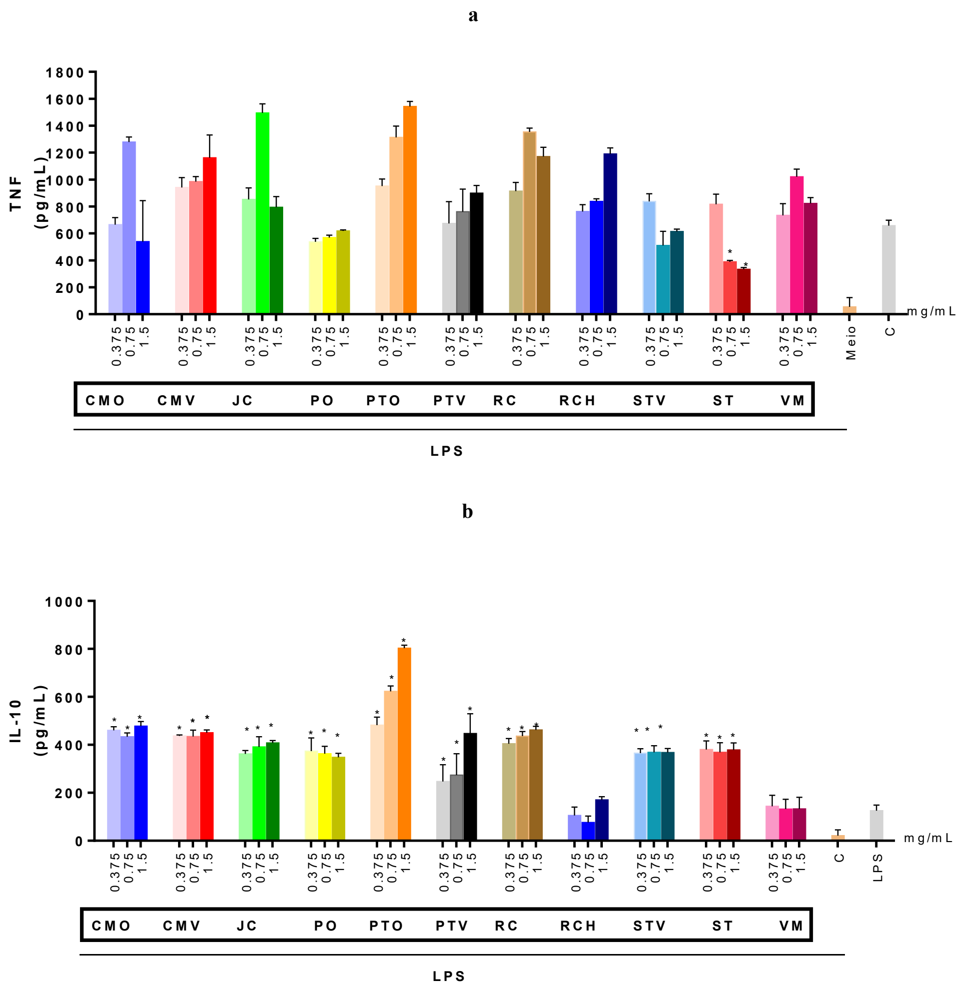
The RC extract demonstrated AA activity by scavenging free radicals (Figure 1C), which may be one of the mechanisms of action for NO inhibition. In addition to LPS, NF-κB can be stimulated by ROS (Rani et al., 2016). The ROS-scavenging activity of the RC extract may reduce the NF-κB activation, thereby decreasing the expression of inflammatory cytokines and iNOS.

The JC extract is standardized with quercetin, a flavonoid that has AA and anti-inflammatory activities linked to a hydroxyl present on its B ring with an electron pair (Tang et al., 2019).



CMO and CMV: *Curcubita moschata*; JC: *Juniperus chinensis*; PO: *Peucedanum ostruthium*; PTO and PTV: *Pinellia ternata Breit.*; RC: *Rubus coreanus*; RCH: *Rubus chingii Hu*; STV and ST: S*olanum tuberosum*; VM: *Viola mandshurica*; NO: nitric oxide, LPS: lipopolysaccharide.

Figure 2. NO production in RAW 264.7 cells. The difference between the extracts was evaluated by one-way ANOVA, followed by a Bonferroni post-test. Significant differences were considered *P < .05, compared to LPS (Control). Results are represented by the mean ± standard deviation. The X-axis indicates the tested concentrations of the aqueous extracts; the Y-axis indicates the NO concentration.



CMO and CMV: *Curcubita moschata*; JC: *Juniperus chinensis*; PO: *Peucedanum ostruthium*; PTO and PTV: *Pinellia ternata Breit.*; RC: *Rubus coreanus*; RCH: *Rubus chingii Hu;* STV and ST: *Solanum tuberosum*; VM: *Viola mandshurica*; IL-10: interleukin-10; TNF-α: tumor necrosis factor-alpha; LPS: lipopolysaccharide.

Figure 3. IL-10 and TNF-α production in RAW 264.7 cells. The difference between the aqueous extracts was evaluated by one-way ANOVA, followed by a Bonferroni post-test. Differences were considered significant at *p < .05, compared to LPS (Control). The results are represented by the mean ± standard deviation. The X-axis indicates the tested concentrations of the extracts; the Y-axis indicates the concentration of cytokines. (A) TNF-α concentration; (B) IL-10 concentration.

Quercetin can suppress NF-κB and block the translocation of subunits, preventing the production of pro-inflammatory cytokines and iNOS (Tang et al., 2019; Yahfoufi et al., 2018). A study evaluating the anti-inflammatory activity of oils derived from JC reported a significant reduction in TNF-α and IFN-γ levels, in addition to demonstrating the greatest ability to decrease IL-1β among the oils tested (Darwish et al., 2019).

3.2.2 Measurement of cytokines in cells treated with phytotherapeutic plants

The results for TNF-α did not follow the same inhibitory pattern observed for NO production (Figure 3A). All plant extracts, at all tested concentrations, stimulated the production of the anti-inflammatory cytokine IL-10, except for the RCH and VM extracts (Figure 3B).

The PTO showed the highest stimulation of IL-10, increasing production by more than 4×. Along with PTV, it also exhibited a dose-dependent response, which is advantageous as it allows effective activity at lower extract concentrations.

As both extracts are derived from the same species and are standardized with tannins, it is suggested that the preferred anti-inflammatory mechanism of these extracts is the stimulation of anti-inflammatory cytokines and that it occurs through the action of the phenolic compounds present. The findings in the literature present positive results regarding the anti-inflammatory activity of isolated tannins (BenSaad et al., 2017) and other species that have tannins in their composition (Xiao et al., 2017); studies regarding *P. ternata* species evaluating anti-inflammatory activity are scarce (Mao & He, 2020). In this study, we demonstrated that *P. ternata* exhibits a strong ability to stimulate the production of anti-inflammatory cytokines, in addition to showing AA potential by reducing reactive species, making it a promising candidate for further investigation.

The RCH and VM extracts could not stimulate IL-10 production at any concentration evaluated (Figure 3B). The species have results consistent with those of the present study regarding NO in the evaluation of isolated compounds (Zha et al., 2011; Zhang et al., 2015a; 2015b). Therefore, the result obtained in the present study suggests that *R. chingii* Hu and *V. mandshurica* act as anti-inflammatories by inhibiting pro-inflammatory cytokines, and not by stimulating anti-inflammatory cytokine production.

In vitro studies present limitations, and polyphenols may suffer some chemical modifications during digestion and absorption, which could alter the results in an *in vivo* model (Bouayed et al., 2011). Although further analyses are necessary to indicate the mechanisms of action and the activity against other cytokines, the results obtained and the reported scarcity in the literature justify the relevance of these species.

4 CONCLUSION

Our results indicate that the herbal extracts studied possess both AA and anti-inflammatory activities, particularly the *Pinellia ternata* Breit. extract. (PTO). *P. ternata* exhibited the strongest AA activity in electron transfer-based methods and induced the highest stimulation of anti-inflammatory cytokine production. Although it was not the extract responsible for the greatest reduction in NO levels, it was still able to decrease this inflammatory mediator, demonstrating bioactivity through multiple mechanisms and making it particularly promising.

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