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Lupinus mutabilis Sweet (*Tarwi*) γ-conglutin Modulates TNF-α and IL-1α Transcriptional Expression in Human Monocytes Infected with *Leishmania* (*V.*) *peruviana*

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ABSTRACT

Background: γ-Conglutin is a hemagglutinating lectin found in the seeds of the Andean legume *Lupinus mutabilis*. *Leishmania* (*Viannia*) *peruviana* is the etiological agent of cutaneous leishmaniasis, which is endemic in Peru. Leishmaniasis is a neglected disease that lacks an effective and non-toxic treatment. The aim of this study was to evaluate the effect of γ-conglutin from *L. mutabilis* Patón Grande ecotype (CoPG) on human monocyte cultures before infection with *L.* (*V.*) *peruviana* and to determine the production of molecules that stimulate cellular immunity.

Methods: CoPG was isolated by chromatography from a hexane extract of *L. mutabilis* seeds. Monocytes were obtained by density gradient centrifugation. The effect of CoPG on their viability was determined by the MTT assay. To assess superoxide and nitric oxide production, monocyte cultures were pre-treated with CoPG and then infected with *L.* (*V.*) *peruviana*. Transcriptional expression of TNF-α and IL-1α in CoPG-treated and parasite-infected monocytes was determined by RT-PCR.

Result: CoPG retained its hemagglutinating activity throughout the isolation process. The purified protein had a molecular weight of 47 KDa. Besides, CoPG at a 20 µg mL^{-1} induced the production of superoxide on the treated monocytes. This CoPG concentration also promoted the overexpression of IL-1α and TNF-α on the infected monocytes. The present study showed that CoPG has a novel *in vitro* immunomodulatory effect. The discovery of bioactive proteins from *L. mutabilis* may uncover a wide range of new applications for this Andean plant.

Key words: Hemagglutinating γ-conglutin, Immunomodulation, *Lupinus mutabilis, Leishmania* (*V.*) *peruviana,* Transcriptional expression.

INTRODUCTION

Lectins isolated from legumes and other plants have an affinity for carbohydrates that allows them to bind glycoconjugates present on cell surfaces, causing agglutination, signalling, recognition or modulation of the cellular response and have therefore become key resources for biomedical research (Perazzo *et al*., 2021).

Lupinus mutabilis is the only domesticated lupin in the Americas and its seeds are gaining importance in the pharmacological field due to the anti-inflammatory properties of its alkaloids (Castañeda *et al*., 2002) and the antioxidant properties of its polyphenols (Pascual-Chagman *et al*., 2021). The protein content of this seeds is composed of globulins and albumins (Suca and Suca, 2015). Among these proteins, γ-globulin (lectin) stands out due to its binding capacity to sugars and rabbit erythrocyte sugar moieties (Carnero *et al*., 2023).

Leishmania spp. are intracellular microorganisms that cause cutaneous (CL) or visceral leishmaniasis. The World Health Organization estimates that approximately 95% of CL cases occur in South America, the Mediterranean region, the Middle East and Central Asia (Abadías-Granado *et al*., 2021). *Leishmania (Viannia) peruviana* is one of the etiological agents for leishmaniasis in the Peruvian Andean region (Zorrilla *et al*., 2017, MINSA, 2022).

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Peru has great biotechnological potential due to its megadiversity. Many legumes, including *L. mutabilis*, contain bioactive molecules such as lectins, which are potent immunomodulators. Treatment of T lymphocytes with phytohemagglutinin (PHA) induces activation signals and stimulates the production of cytokines such as TNF-α, IFN-γ, IL-6 (Zivancevic-Simonovic *et al*., 2022) and stimulates the

immune response against infections caused by *Leishmania* spp. (Jebali *et al.*, 2014; Jandú *et al.*, 2017).

Given the importance of discovering new uses for lectins, the aim of this study was to evaluate the effect of γ-conglutin from *L. mutabilis* ecotype Patón Grande (CoPG) on human monocyte cultures prior to infection with *L. (V.) peruviana*. Availed parameters included the production of superoxide and nitric oxide and the transcriptional expression of tumor necrosis factor alpha (TNF-α) and interleukin-1 alpha (IL-1α).

MATERIALS AND METHODS

The experiments were conducted in the Faculty of Biological Sciences of Universidad Nacional Mayor de San Marcos between 2018 and 2019. Seeds of *Lupinus mutabilis* were collected in the district of Usquil, province of Otuzco (S7°54′0″ W78°34′59.99″), La Libertad region, Peru. The agronomic study and classification of the ecotype Patón Grande (Accession Code: AC TLM 12) was carried out at the Grain Legumes and Oilseeds Program of the Universidad Nacional Agraria La Molina, according to the parameters established by Camarena *et al.* (2013). Isolation and characterisation of CoPG was performed according to the method described by Carnero *et al.* (2023). The rabbit erythrocyte hemagglutination (HA) test was performed at each step to keep track of any loss of activity during purification.

Human monocytes isolation from peripheral blood

The double gradient method of Menck *et al.* (2014) was slightly modified. Heparinised venous blood was obtained from a single donor (healthy male volunteer, age 25 years) with prior informed consent. The blood was centrifuged (150 × g, 5 min). The cell pellet was resuspended in phosphate buffered saline (PBS) with 2% fetal bovine serum (PBS) in a 1:1 ratio. The resuspended cell pellet (4 mL) was layered over 5 mL lymphocyte separation medium (density: 1.077 g mL^{-1}) and centrifuged (4000 × g, 30 min). Peripheral blood mononuclear cells (PBMC) ring was collected and washed twice with 1X PBS- 1mM EDTA (pH 7.4). Third wash was performed with RPMI supplemented with 10% FBS (coRPMI). The pellet was resuspended in 1 mL of 1X PBS (pH 7.4).

An isosmotic 46% Percoll solution (density 1.131 g mL^{-1}) was used as second gradient. Briefly, 4 mL PBMC were added to 46% Percoll (5 mL) and centrifuged (550 × g, 30 min). The white ring of monocytes was collected from the 1X PBS - 46% Percoll interface and washed twice with 1 X PBS-1 mM EDTA (400 g, 10 min). The cell pellet was suspended in coRPMI. Cell viability and counts were determined using trypan blue in a Neubauer counting chamber. The monocytes (2×10^5) were added to each well (100 µL/well) of 96-well microplates and cultivated for 12 h at 37°C, under standard culture conditions (5% CO_2 and 95% relative humidity). Monocyte adhesion was assessed by microscopy and the cells were washed twice with 1X PBS before being cultured in coRPMI for subsequent assays.

Effect of CoPG on monocyte viability

This was assessed by adding CoPG concentrations of 5, 10 and 20 µg mL^{-1} (diluted in coRPMI; n=3). Monocyte cultures were then incubated under standard conditions for 24 h (37°C). Following, 15 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent were added and incubated (2 h) under standard conditions. The supernatant was discarded and 140 µL dimethyl sulfoxide (DMSO) were added to each well, plates were incubated with agitation (50 rpm, 10 min) and absorbance was measured at 492 nm with a 630 nm reference filter. PHA was used as positive control.

Leishmania (*V.*) *peruviana* promastigotes

The determination of the stationary phase in the growth curve was performed using promastigotes from a reference strain (MHOM/PE/84/LC26) donated by the National Institute of Health (Peru). Viability and promastigote counts were assessed every 12 h in a Neubauer chamber using trypan blue (n=3). Promastigotes were cultured in coRPMI at 26°C (under standard culture conditions).

Infection time of monocytes with *L.* (*V.*) *peruviana* promastigotes

Monocytes were co-cultured with promastigotes in stationary phase for 6, 18 and 24 h at 26°C (under standard culture conditions) (n=3) and the number of intracellular amastigote in each monocyte was determined. For this, 1 mL of monocytes (2×10^5/mL) suspended in coRPMI were distributed on coverslips placed in Petri dishes (35 × 10 mm) and incubated for 12 h at 37°C (under standard culture conditions). The coverslips were then washed twice with 1X PBS and 1 mL of stationary phase promastigotes (2×10^6/mL) (1 monocyte:10 promastigotes) was added (Bonyek-Silva *et al.*, 2021). Coverslips were washed with PBS 1X and stained with Giemsa. The percentage of infection was determined by counting 100 total monocytes (infected with amastigotes and uninfected) by observing the coverslips under a light microscope (× 1000). The number of infected monocytes was used to calculate the percentage of infection.

Superoxide production

In a 96-well microplate, 100 µL of monocyte suspension (2×10^5 mL^{-1}) and 100 µL of CoPG concentrations (5, 10 and 20 µg mL^{-1} in coRPMI) (n=3) were added. Untreated monocytes and PHA-treated (50 µg mL^{-1}) were used as controls. Treated cells were incubated for 24 h at 37°C (under standard culture conditions). Then, 20 µL of nitroblue tetrazolium reagent (Sigma Aldrich) was added for 4 h at 37°C (under standard culture conditions). The microplate was washed with 1X PBS to remove unattached monocytes and 100 µL of absolute methanol were added (3 min). The microplate was placed to dry at room temperature. Formazan was dissolved in 140 µL DMSO and 120 µL KOH (2 M). The microplates were placed on a shaker (50 rpm, 10 min) for 1 h. The absorbance was read at 630 nm.

Nitric oxide production (NO)

Cultures were performed in a similar manner as described for superoxide. The supernatant was collected and processed according to the instructions of the Griess reagent kit (Promega) (n=3). Absorbance was read at 492 nm with a 630 nm reference filter. Quantification was performed using the values of the sodium nitrite (100 µM mL^{-1}) reference curve.

Stimulation with CoPG in co-cultures

The concentration of CoPG (20 µg mL^{-1}) that induced the highest superoxide production in monocyte cultures was used for stimulation of monocyte cultures infected with *L. (V.) peruviana* (co-cultures) (n=3). Before infection, 50 µL monocyte suspension (2×10^5 mL^{-1}) was stimulated with 50 µL CoPG (MLeCoPG) for 24 h as described. Controls were infected monocytes treated with 50 µg mL^{-1} PHA (MLePHA), untreated infected monocytes (MLe) and untreated, uninfected monocytes (M). They were incubated for 24 h at 37°C (standard culture conditions) and superoxide and NO production were determined as described.

Transcriptional expression of proinflammatory cytokines

The transcriptional expression (TE) of IL-1α and TNF-α was evaluated by conventional reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from MLeCoPG cultures and controls (MLePHA, MLe and M) according to the manufacturer's instructions (GeneJET Plant RNA Purification kit, Thermo Scientific™ K0801). RNA integrity was analysed by 1.5% agarose gel electrophoresis in 1X TAE buffer at 100 V (60 min), quantified by fluorometry using the Qubit 3.0 kit (Invitrogen) and stored at -80°C until use. The cDNA was prepared using the Maxima First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific™ #K1641). The cDNA was quantified using the Qubit 3.0 kit and used immediately.

Primers for IL-1α, TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH endogenous control) were used in the RT-PCR. They were designed using the Primer BLAST software.

Primers for IL-1α were:
F 5′CTTCATTGCTCAAGTGTCTGAA3′,
R 5′GACAGGATATGGAGCAACAAGT3′;
for TNF-α:
F 5′GTCAGATCATCTTCTCGAACC3′,
R 5′CAGATAGATGGGCTCATACC3′; and
for GADPH:
F 5′TCCCTGAGCTGAACGGGAAG3′,
R 5′GCAGGAGTGGGTGTCGCTGT3′ (F: forward, R: reverse).

The Platinum® PCR SuperMix was used for amplification according to the manufacturer's instructions (Invitrogen cod. 11306-016). The protocol for amplification of cytokines and endogenous control was: initial denaturation (94°C, 5 min), final denaturation (94°C, 30 s), annealing (57°C, 30 s), initial extension (72°C, 45 s) and final extension (72°C, 7 min). The amplicons were subjected to electrophoresis in 1.5% agarose (100 V, 60 min) using TAE 1X buffer, gels were analysed with a gel imager. The ratios of mRNA densities for IL-1α to GAPDH mRNA density were analysed using ImageJ software (NIH, USA). The same procedure was used for TNF-α.

Statistical analysis

Data for monocyte viability, *L.* (*V.*) *peruviana* growth curve, monocyte infection times, NO and superoxide production were analysed using GraphPad Prism software version 8.0. Data were expressed as mean ± standard error of the mean. A p-value less than 0.05 was considered statistically significant. Dunnet's multiple comparison test was used to compare treatments with control (M). Tukey's multiple comparison test was used to compare results between treatments.

RESULTS AND DISCUSSION

Isolation of CoPG and HA capacity

The purification protocol described by Carnero *et al.* (2023) was successfully replicated. The gross protein yield was ~200 mg/100 g of hexane-defatted flour and, the purified protein had a yield of ~ 0.5 mg/100 g flour. It is evident that the chemical extraction process used to obtain the CoPG significantly reduced the amount of protein in the flour, as the used treatment prioritize functionality of soluble proteins.

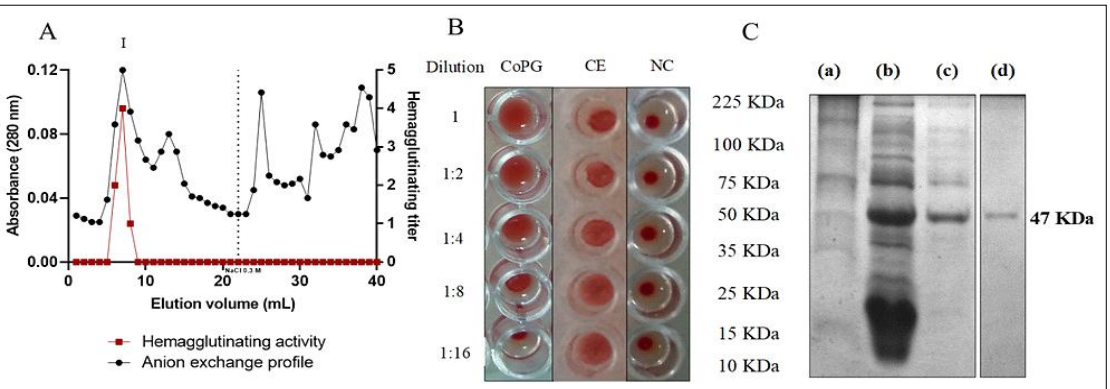
CoPG with HA capacity was present in the first peak of the chromatogram obtained by anion exchange chromatography (Fig 1A), with HA titers of 8 (0.31 HA units) (Fig 1B). These findings are similar to previous reports for lectin isolation from *L. mutabilis* seeds (Carnero *et al.*, 2023). Regarding the HA titre for the crude extract (64), Falcón *et al.* (2000a) reported a higher titre (512) for *L. mutabilis*, but they did not specify the ecotype. Falcón *et al.* (2000b) showed that the purified lectin from *Lupinus albus* did not have the HA activity present in the extract and concluded that this species has at least two different lectins. This is important because the overall biochemical composition and bioactivity vary depending on the climatic conditions in which the different ecotypes develop (Camarena *et al.*, 2013).

Molecular weight of CoPG

The crude PG extract showed bands with molecular weights (MW) between 10 and 225 kDa. CoPG had a MW of ~47 kDa (Fig 1C), similar to the 46 kDa γ-conglutinin isolated by Carnero *et al.* (2023).

Effect of CoPG on monocyte viability

The viability of the isolated monocytes was 90%. No cytotoxic effect was observed in monocyte cultures at the evaluated CoPG concentrations, indicating that the product is safe. No other related reports were found. Increased cell viability was observed in the PHA control. This finding suggests that PHA (50 µg mL^{-1}) stimulates mitosis in adherent monocytes, as observed by Greenwood (1977) in scar monocytes.



(A): Chromatographic profile of the fractions obtained by anion exchange chromatography, HA was present in peak I. (B) CoPG presented HA up to a titer of 8 and the crude extract (CE) had a titer of 64. (C) MW of CoPG was ~47 KDa (lane d), this band was present in the crude extract (lane b) and in the fractions obtained by exclusion chromatography (lane c). NC: Control, no HA.

Fig 1: Characterization of γ-conglutinin from *Lupinus mutabilis* ecotype Patón Grande (CoPG).

Salaberria *et al.* (2013) observed in an *in vivo* model (passerine birds) that the PHA-induced inflammatory response is not only a consequence of the activation of T lymphocytes, but also of monocytes.

Growth curve of promastigotes from *L.* (*V.*) *peruviana*

The logarithmic phase of the promastigotes occurred between day 1 and 2 of culture. The stationary phase started on day 2.5 and lasted until day 5.5 (Fig 2A). Between days 1 and 3, promastigotes showed high mobility and elongated bodies. Starting day 4, their mobility decreased significantly and they acquired their characteristic rounded shape. Calvay-Sánchez *et al.* (2015) observed that the stationary phase started on day 7 when seeding 5×10^2/mL^{-1} *L.* (*V.*) *peruviana* promastigotes (29°C) in complete Schneider medium and lasted for 12 days. Growth curves vary according to *Leishmania* species, promastigote inoculum, incubation temperature and culture medium. It should be noted that the culture medium used also depends on the specific purpose of the research, for example RPMI is used for long-term maintenance of parasites and Schneider for the study of metacyclogenesis (Siripattanapipong *et al.*, 2019).

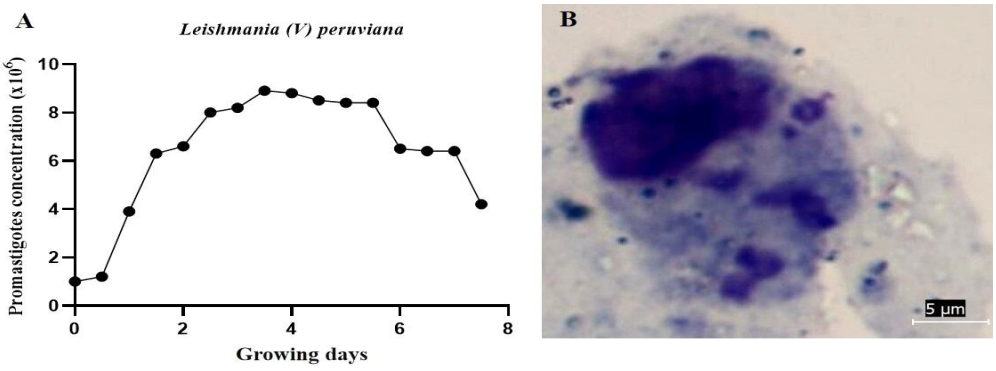
Infection of human monocytes by *L.* (*V.*) *peruviana*

At 6 and 18 h the percentage of infected monocytes was below 50%, at 24 h, 80% of the monocytes had at least one amastigote or were invaded by them (Fig 2B). The time of infection of monocytes depends on the *Leishmania* species and the Toll-like receptors (TLRs) capable of recognising the parasite. Viana *et al.* (2018) observed that monocytes infected with *L. braziliensis* (6×10^7 parasites/mL) 16 h post-incubation showed higher expression of TLR2 and TLR9 compared to *L. infantum*. In the present study, the infection time of monocytes was longer (24 h), which could be due to the lower inoculum of promastigotes (2×10^6 mL^{-1}). It is also possible that those monocytes have a high level of expression of the CD11c molecule on the membrane (Loria-Cervera and Andrade-Narvaez, 2020). No similar reports have been found for *L. (V.) peruviana* or on the interaction of CD11c with *L. mutabilis* lectin.

Superoxide production

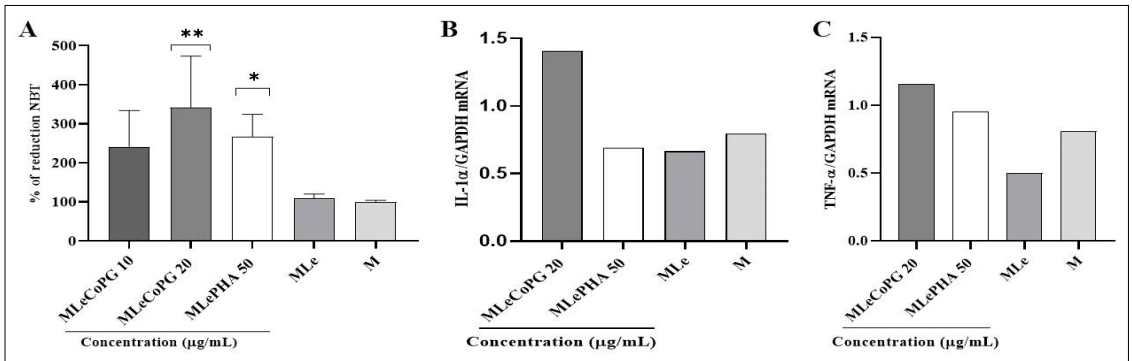
A higher amount of superoxide radical was observed in monocyte cultures pre-treated with 20 µg mL^{-1} CoPG. The result was similar for CoPG-pre-treated and infected (MLeCoPG) and PHA-treated monocytes compared to controls MLe and M ($p=0.002$ and $p=0.02$ respectively) (Fig 3A). No differences were found between MLe and M. Increased superoxide production by MLeCoPG suggests a preventive and stimulatory function of CoPG. These properties have also been reported for lectins such as KM (+) (Artin M) from *Artocarpus integrifolia*, which increased resistance and induced IL-12p40 production by BALB/c macrophages, modulating the conversion of type 2 to type 1 macrophages capable of responding to *Leishmania* antigens (Panunto-Castelo *et al.*, 2001). Thomazelli *et al.* (2018) demonstrated that pre-treatment of PBMC with concanavalin A stimulated the production of pro-inflammatory (IFN-γ, TNF-α, IL-2 and IL-6) and anti-inflammatory (IL-4 and IL-10) cytokines, reactive oxygen species and inducible nitric oxide synthase without nitric oxide production.

The two main mechanisms responsible for the control and elimination of *Leishmania* are the production of reactive oxygen species (ROS) generated by phagocytic responses and NO generated by inducible nitric oxide synthase, which requires the prior production of IFN-γ (Thomazelli *et al.*, 2018). Scott and Novais (2016) reported that human and mouse monocytes infected with different *Leishmania* species produce high levels of ROS, however the parasite can inhibit ROS production in phagolysosomes. For this reason, the present study used monocytes previously stimulated with CoPG and showed that this treatment increased ROS production compared to the MLe assay, suggesting that *L. (V.) peruviana* caused a blockage or reduction of phagocytic activity in MLe.



The logarithmic phase occurred between days 1 and 2. The stationary phase began on day 2.5 and ended on day 5.5. Photomicrograph showing parasite amastigotes inside human monocytes after day 1 of co-culture (x 1000). Results are the mean of the number of promastigotes at the times evaluated ± SD in triplicate wells.

Fig 2: Growth curve of *L.* (*V.*) *peruviana* in coRPMI.



(A) Higher superoxide production was observed in MLeCoPG and MLePHA (**p=0.002 and *p=0.02, respectively). (B y C). Higher transcriptional levels for the 2 cytokines were produced in the MLeCoPG. MLePHA alone induced transcriptional expression of TNF-α. Densitometric quantification was performed using ImageJ software. MLeCoPG: CoPG-pretreated monocytes infected with *L. (V.) peruviana* (Le); MLePHA: PHA-pre-treated and infected monocytes; MLe: Untreated and infected monocytes; M: Untreated and uninfected monocytes.

Fig 3: Superoxide and proinflammatory cytokine.

Oxide nitric production

No significant NO production was found in any of the experiments (p>0.05). Its induction depends on IFN-γ produced by T-helper 1 (Th 1) and NK cells, which are not present in the model used in this study.

Transcriptional expression of IL-1α and TNF-α in infected monocytes

CoPG induced a higher transcriptional level for the 2 cytokines compared to MLe and M controls (Fig 3B, 3C), whereas only TNF-α expression was present in the PHA treatment (50 µg mL^{-1}) (Fig 3C). An important aspect of the IL-1 family of cytokines is that, together with IL-12, IL-1, IFN-γ, TNF-α and IL-2, they are part of the Th1 profile and are responsible for controlling parasite proliferation and dissemination, promoting granuloma formation (Cavalli *et al.*, 2021). The identification of cytokines during the infection process is important because they are currently considered targets for the treatment of CL (Da Silva *et al.*, 2020) and can be detected in assays using lectins from legumes or other plants. In contrast to the stimulatory effect on proinflammatory cytokine production found for CoPG, assays using γ-conglutin from *Lupinus angustifolius* in an induced inflammation cell model revealed a strong reduction in cellular oxidative stress, resulting in decreased nitric oxide synthesis and inducible nitric oxide synthase (iNOS) transcriptional levels, improved glucose uptake and decreased proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-6, IL-12, IL-17 and IL-27 (Lima-Cabello *et al.*, 2020).

Regarding cytokine production as a result of *Leishmania* infection, Viana *et al.* (2018) demonstrated lower TNF-α production and lower TNF-α/IL-10 ratio in monocytes infected with *L. infantum* compared to those infected with *L. braziliensis*; consequently, the low inflammatory profile resulted in 100-fold lower protective efficacy. In the present study, the finding of increased transcriptional levels for IL-1α and TNF-α in monocytes pre-treated with CoPG represents a starting point for the investigation of other cytokines of the Th1 profile and their antagonists. No reports

have been found on the activity of other legume lectins in monocytes infected with *L.* (*V.*) *peruviana*.

Based on the results of this investigation, further research is suggested, including the evaluation of other pro- and anti-inflammatory cytokines, the inclusion of another endogenous gene in RT-PCR or RT-qPCR and stimulatory controls of cytokine production such as lipopolysaccharide and CoPG pre-treatment of other phagocytic cells involved *in vivo* response (neutrophils and dendritic cells). Potential studies include the use of CoPG-coated nanoparticles, which have shown good leishmanicidal efficacy with peanut lectin and *L. major* infected mouse macrophages (Jebali *et al.*, 2014). This is the first report on the utility of CoPG and predicts this lectin as a promising resource with the potential to join the 'glycan decoder' lectins widely used in glycomics (Hu *et al.*, 2015).

CONCLUSION

In the present study, CoPG was shown to stimulate the production of superoxide (ROS), which influences the increased transcriptional expression of the proinflammatory cytokines IL-1α and TNF-α in human monocytes infected with *L. (V.) peruviana in vitro*. CoPG has also been shown to stimulate the production of superoxide and the aforementioned cytokines involved in the innate pro-inflammatory response, thereby generating a protective profile against the parasite. However, it is necessary to continue studying the characteristics of the monocyte receptors that interact with CoPG and to develop strategies that allow its use for the topical treatment of CL, especially in patients with diseases that require the use of immunosuppressants such as anti-TNF-α. In addition, its use as an adjuvant in experimental vaccines against *L. (V.) peruviana*, its role in co-infections and the ligands with which it may interact need to be evaluated to help identify new pharmacological targets in diseases such as cancer. Ultimately, this knowledge will add value to *L. mutabilis* seed.

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Conflict of interest

The authors report there are no competing interests to declare.

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