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SHORT COMMUNICATION

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GPETAFLR, a biopeptide from *Lupinus angustifolius* L., protects against oxidative and inflammatory damage in retinal pigment epithelium cells

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Abstract

GPETAFLR, an octapeptide released from the enzymatic hydrolysis of lupine (*Lupinus angustifolius* L.) protein, has demonstrated anti-inflammatory effect in myeloid lineage. This work aims to evaluate in retinal pigment epithelium (RPE) cells the protective role of GPETAFLR on both oxidative and inflammatory markers known to be involved in age-related macular degeneration (AMD). In comparison with stimulated control cells, GPETAFLR increased glutathione production and diminished the secretion and gene expression of VEFG, IL-1β, IL-6, IFNγ, and TNF-α, as well as reactive oxygen species, and nitrite output. Our findings reveal that GPETAFLR, a novel plant peptide, is able to protect against RPE oxidative stress and inflammation. Taken together, these results strongly support innovative nutritional strategies considering *Lupinus angustifolius* L. as source of proteins to prevent the onset and progression of AMD.

Practical applications

We reveal a novel nutraceutical impact of GPETAFLR peptide in human RPE cells to prevent oxidative and inflammatory mediators. Our results support that the intake of *Lupine angustifolius* L., proposed to be a reservoir of GPETAFLR, could lessen the functional decay of RPE cells, leading therefore to a slowdown of the progress of AMD during age. Not only this work, but also future simple clinical studies should raise new nutritional strategies focused on understanding the etiological role of the foods, nutrition, and metabolism in the pathogenesis of ocular disorders.

KEYWORDS

age-related macular degeneration, biopeptide, lupine seeds, *Lupinus angustifolius*, retinal pigment epithelium

1 | INTRODUCTION

Proteins from plants have raised as an option for the isolation of bioactive peptides (Lee & Hur, 2017; Pihlanto, Mattila, Makinen, & Pajari, 2017). These biopeptides are short amino acid sequences, inactive in the native protein, that are released by digestive enzymes and then reach systemic circulation as bioactive molecules (Millan-Linares, Millan, Pedroche, & Yust, 2015). Recently, our laboratory has demonstrated the presence of a novel octapeptide isolated

Abbreviations: AMD, age-related macular disease; BRB, blood–retinal barrier; CNV, choroid neovascularization; GSH, glutathione; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; NO, nitric oxide; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RPE, retinal pigment epithelium; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

from *Lupinus angustifolius* L. protein hydrolysates. As depicted in Figure 1, the amino acidic sequence was described as glycine–proline–glutamate–threonine–alanine–phenylalanine–leucine–arginine (GPETAFLR) with α-helix three-dimensional structure (protein structure model was generated by the automated SWISS-MODEL homology modeling pipeline, Bienert et al., 2017). Previously, antioxidative and anti-inflammatory properties of GPETAFLR have been tested in a human myeloid lineage (Millan-Linares et al., 2018). GPETAFLR showed decrease the expression of pro-inflammatory cytokines as TNF-α or IL-1β and increase those that participate in the anti-inflammatory response as IL-10 or IL-4 in THP-1-derived macrophages (Millan-Linares et al., 2015), monocyte-derived osteoclasts (Millan-Linares et al., 2018), and primary human monocytes (Montserrat-de la Paz et al., 2019).

Nutrition, age, oxidative stress, or a state of chronic inflammation, among other causes, may induce dysfunction and degeneration of retinal pigment epithelium (RPE) (Levy et al., 2015). RPE is a monolayer of pigmented epithelial cells located between the retina and choroid that plays a critical role in maintaining visual function and thereby in the pathogenesis of retinal degenerative diseases (Pang, Zhou, & Kuang, 2018). RPE regulates the volume and chemical composition of the subretinal space and nutrient transport between the retina and choroid. Therefore, RPE functions as blood–retinal barrier that protects the health and integrity of the retina and choroid (Qin et al., 2017; Sonoda et al., 2009). Recent studies are helping to clarify the role of RPE cells in the immune and inflammatory response of the retina (Montserrat-de la Paz et al., 2016). The loss of integrity and functionality of RPE cells involves irreversible damage to the photoreceptors and consequently a loss of central vision. This is known as age-related macular degeneration (AMD) (Montserrat-de la Paz, Naranjo, Bermudez, et al., 2017), which is the leading cause of blindness and its prevalence continues to increase in parallel with the increase in life expectancy aging and change in lifestyle of the world population (Montserrat-de la Paz et al., 2016).

Therefore, this study aims for the first time the role of GPETAFLR, isolated from lupine (*Lupinus angustifolius* L.), on oxidative stress and inflammation in RPE cells.

2 | MATERIAL AND METHODS

2.1 | Chemicals

The following ELISA kits were purchased from Diaclone Research (Besancon, France): VEGF, IFNγ, TNF-α, IL-6, and Human IL-1β. Hydrogen peroxide (H_2O_2) was purchased from Panreac (Barcelona, Spain). Other chemicals were purchased from Sigma-Aldrich Chem. (St. Louis, MO, USA).

2.2 | GPETAFLR synthesis

GPETAFLR, originally isolated from lupine (*Lupinus angustifolius* L.) protein hydrolysates (Millan-Linares et al., 2015; Millan-Linares, Yust, Alcaide-Hidalgo, Millan, & Pedroche, 2014), was finally synthesized at 95% purity, measured by HPLC-UV at 220 nm, following Fmoc solid-phase method by the Barcelona Scientific Park Foundation (Barcelona, Spain).

2.3 | ARPE-19 cell culture

Dr. Edilberto Ojeda (UPV/EHU) kindly provided ARPE-19 (human RPE cells). Cells were cultured ay 37°C/5% CO_2 in a medium resulting from the mixture of DMEM and F12 (1:1) (Life Technologies) completed with 100 U/mL penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Lonza) and 10% fetal bovine serum (Hyclone). Every 3–4 days, subcultures were made using 0.25% trypsin-EDTA (Montserrat-de la Paz et al., 2016). Cells were passaged before coming to confluence, in order to keep them in an undifferentiated state. For the experiments we used ARPE-19 cells at 5–10 passages.

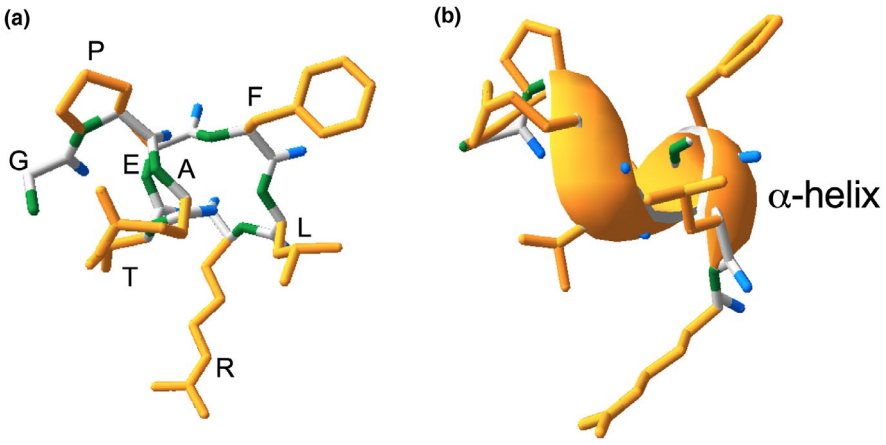

FIGURE 1 Chemical (a) and secondary three-dimensional (b) structure of GPETAFLR peptide, an octapeptide isolated from *Lupinus angustifolius* L., which amino acid sequence is identified as: Glycine (G), Proline (P), Glutamate (E), Threonine (T), Alanine (A), Phenylalanine (F), Leucine (L), and Arginine (R). Yellow color was used to side chain, green color was used to amino group, and blue color was used to carboxyl group

2.4 | Cell viability assay (MTT)

The chemical reduction to formazan of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is the basis of the colorimetric assay used to determine ARPE-19 cells viability. Briefly, after seeding in 96-well plates 105 ARPE-19 cells/well, the culture was incubated during 24 hr in the presence of concentrations of the peptide GPETAFLR, between 50 μg/ml and 100 μg/ml, then 0.5 mg/ml of MTT in aqueous solution was added to continue incubation 2 hr at 37°C (Montserrat-de la Paz, Naranjo, Lopez, et al., 2017). In order to estimate cell viability, a Multiskan Spectrum plate reader (ThermoLab systems) was used to quantify the % of absorbance at 570 nm wavelength compared to a 620 nm reference.

2.5 | Intracellular reactive oxygen species (ROS)

The intracellular ROS was determined using the CellROX Green Reagent (ThermoFisher Scientific, Madrid, Spain). After in vitro stimulation with H_2O_2 at 100 μM, ARPE-19 cells were exposed to 50 μg/ml and 100 μg/ml of the peptide GPETAFLR for 24 hr and then with CellROX Green Reagent (5 μM) for 30 min. Cells were washed with phosphate-buffered saline and fixed with 3.7% formaldehyde, and the fluorescence signal was analyzed in a Fluoroskan Microplate Fluorometer (ThermoFisher Scientific) equipped with a 485/555 excitation/emission filter set. The autofluorescence of cells was measured under the same conditions but without adding CellROX Green Reagent (Lopez et al., 2017). Data shown refer to the % of intracellular ROS production and to the comparison with a positive control (100% ROS production) after cell treatment in the presence of H_2O_2.

2.6 | Glutathione (GSH) assay

After in vitro stimulation with H_2O_2 at 100 μM, ARPE-19 cells were exposed to 50 μg/ml and 100 μg/ml of the peptide GPETAFLR for 24 hr. Cell extracts were obtained in 5% sulfosalicylic acid followed by two freeze/thaw cycles (Yan, Liang, Li, & Zheng, 2015). GSH was determined in samples of cell extracts by measuring the formation of *p*-nitrophenol from 5,5′-dithiobis (2-nitrobenzoic acid) in the presence of GSH reductase and the reduced form of nicotinamide adenine dinucleotide phosphate according to the GSH Assay Kit (CS0260; Sigma-Aldrich). Data shown refer to the % of intracellular GSH concentration and to the comparison with a negative control (untreated cells, 100% GSH concentration).

2.7 | Nitrite production

Briefly, after being stimulated with H_2O_2 at 100 μM, an ARPE-19 cell culture (10^5 cells/well in 24-well plates), was incubated for 24 hr in the presence of 50 μg/ml and 100 μg/ml of the peptide GPETAFLR. The Griess reagent (Sigma-Aldrich) was used to calculate the production of nitrite, considered an indication of nitric oxide (NO) generation. Once transferred 100 μl of the culture supernatant to a 96-well plate, a volume of 100 μl of Griess reagent was added (Quilez, Montserrat-de la Paz, De la Puerta, Fernandez-Arche, & Garcia-Gimenez, 2015). A BioTek plate reader measured absorbance at 540 nm wavelength, using a sodium nitrite standard curve to estimate concentration.

2.8 | Cytokine release

After stimulation treatment with 100 ng/ml of lipopolysaccharide (LPS) in vitro, ARPE-19 cells were cultured in 24-well plates at a 10^5 cells/well density and then treated 24 hr with two doses of the peptide GPETAFLR, 50 and 100 μg/ml. ELISA kits were used to calculate in cell culture supernatants several concentrations in pg/ml, from every standard curve: vascular endothelial growth factor (VEGF), IL-1β, IFNγ, IL-6, and TNF-α. A Multiskan Spectrum plate reader measured absorbance at 450 nm wavelength.

2.9 | RNA isolation and quantitative real-time PCR analysis

After in vitro stimulation with LPS at 100 ng/ml, the ARPE-19 cells were cultured in 24-well plates at 10^5 cells/well density, and incubated during 24 hr in the presence of 50 μg/ml and 100 μg/ml of the peptide GPETAFLR. Trisure Reagent (Bioline) was used to extract total RNA. In a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), RNA quality was evaluated by A_{260}/A_{280} ratio. Reverse transcription was performed using 1,000 ng RNA (iScript, BioRad). Twenty nanograms of the cDNA obtained were used as template for real-time polymerase chain reaction amplifications. Amplification of each specific gene product was performed using a CFX96 system (BioRad). Every PCR reaction contained brilliant SYBR green QPCR Supermix (BioRad), the primer pairs for the corresponding gene and cDNA template. As housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18-S ribosomal RNA (18-S) were used Table 1 shows both the sequence and the information for the primers. Every reaction was done in triplicate. To estimate the relative mRNA expression of analyzed genes, the average threshold cycle (Ct) values of the triplicates were used. With the standard $2^{-(\Delta\Delta Ct)}$ method, the magnitude of change of mRNA expression for candidate genes was assessed (Montserrat-de la Paz, Rodriguez, et al., 2017; Naranjo et al., 2016). To determine the relative expression of the studied genes, we used the average of the Ct data of housekeeping samples. Results were normalized using housekeeping genes expression and showed as percentage of control samples.

2.10 | Statistical evaluation

Data in figures and text are expressed as arithmetic mean ± *SD* (standard deviations). Every experiment was performed in triplicate. For evaluation of the results we used Graph Pad Prism Version 6.01 software (San Diego, CA, USA). Significance of parameter variations within treated groups was evaluated by one-way analysis of variance, following Tukey's multiple comparisons test as *post hoc* test. Those *p* values fewer .05 were determined statistically significant.

TABLE 1 Sequences of RT-qPCR primers for gene expression analysis

Target	No. *GenBank*	Direction	Secuence (5′ --> 3′)
GAPDH	NM_001289746	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGTCTCTCT
18-S	NR_003286.2	Forward	GGCCCTGTAATTGGAATGAGTC
		Reverse	CCAAGATCCAACTACGAGCTT
iNOS	NM_000625	Forward	ACCCAGACTTACCCCTTTGG
		Reverse	GCCTGGGGTCTAGGAGAGAC
IL-1β	NM_000576	Forward	GGGCCTCAAGGAAAAGAATC
		Reverse	TTCTGCTTGAGAGGTGCTGA
IL-6	NM_000600	Forward	TACCCCCAGGAGAAGATTCC
		Reverse	TTTTCTGCCAGTGCCTCTTT
TNFα	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
IFNγ	NM_000619	Forward	CAGGCAGGACAACCATTACTGGGATGCTC
		Reverse	TGAACTCATCCAAGTGATGGCTGAACTGTCG
VEGF	NM_001171623.1	Forward	CCCACTGAGGAGTCCAACAT
		Reverse	TTTCTTGCGCTTTCGTTTTT

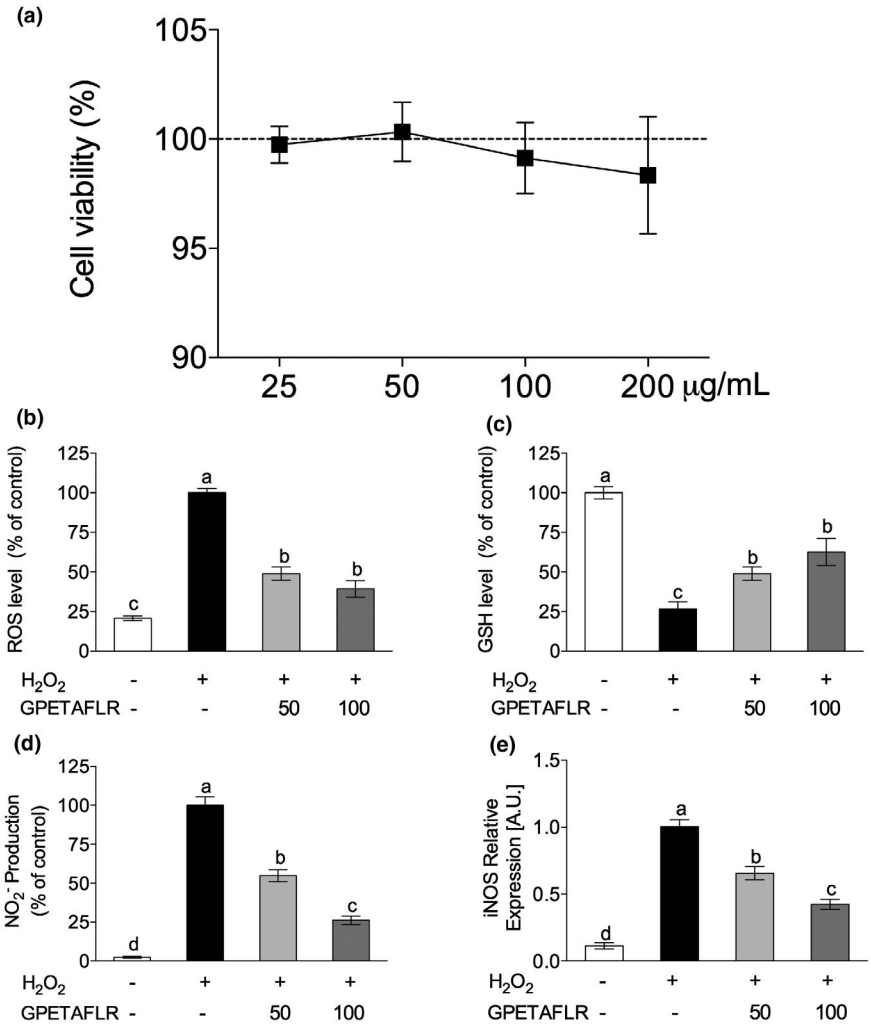

FIGURE 2 Cell viability (a), Intracellular ROS (b), GSH (c), and nitrite (d) production, expressed as percentage of fluorescence/absorbance, and iNOS mRNA levels (e) relative to cells treated with H_2O_2, after the treatment of ARPE-19 cells with GPETAFLR at 50 and 100 µg/ml for 24 hr. Values are presented as means ± *SD* (*n* = 3). Different letters denote statistical differences ($p < .05$)

3 | RESULTS AND DISCUSSION

Treatment during 24 hr with peptide GPETAFLR up to 200 μg/ml doses, had no cytotoxic effect in ARPE-19 cells (Figure 2a). Considering the MTT assay, 50 and 100 μg/ml of peptide showed more than 98% viability of the cells. In previous reports, GPETAFLR were not cytotoxic to primary human monocytes (Montserrat-de la Paz et al., 2019), human monocyte-derived osteoclasts (Millan-Linares et al., 2018), and THP-1 cells (Millan-Linares et al., 2015) at the same concentrations.

Progress of many eye diseases, including AMD, glaucoma, and cataracts, are related to oxidative damage, resulted from excess production of NO or ROS (Yonekawa, Miller, & Kim, 2015). For that reason, quantification of both ROS and NO concentrations in ARPE cells, let us analyze the preventive role of peptide GPETAFLR on oxidative conditions. H_2O_2 remarkably increased both intracellular ROS (Figure 2b) and nitrites (Figure 2d), compared to nonstimulated cells. GPETAFLR decreased ROS production in H_2O_2-stimulated ARPE cells. The relative increase caused by H_2O_2 in nitrite production was higher than that of ROS. In addition, LPS stimulated the iNOS mRNA transcriptional activity which was downregulated by GPETAFLR (Figure 2e). GSH protects against oxidative damage in many tissues, including RPE (Sun, Zheng, Wang, & Liu, 2018). GPETAFLR increased GSH level against oxidative damage in ARPE-19 cells (Figure 2c). We have no evidence of previous studies regarding the effect of a plant peptide on the balance of ROS, NO, and GSH in human RPE cells.

In order to determine possible anti-inflammatory effects on human RPE cells of the peptide GPETAFLR, we studied in ARPE-19 both release and gene expression of IFNγ, IL-1β, TNF-α, and IL-6. As shown in Figure 3, LPS stimulated the transcriptional activity of genes IL-1β (Figure 3a), IL-6 (Figure 3b), TNF-α (Figure 3c), and IFNγ (Figure 3d). GPETAFLR, particularly at 100 μg/ml, showed to produce less inflammatory mediators than LPS-stimulated cells (Figure 4). Present findings in ARPE-19 cells confirm anti-inflammatory effects of peptide GPETAFLR, emphasizing a possible function on acute inflammation. In the literature, it possible to find large number of examples where plant-derived biopeptides are used as anti-inflammatory or antioxidant compounds. One of them is 1,2,3,4,6 penta-O-galloyl-β-D-glucose, a naturally polyphenolic compound present in some medicinal herbs as *Rhuschinensis Mill*. *Fagopyrum tataricum*, commonly known as buckwheat, is another example of bioactive plant. Researchers found that buckwheat extracts may inhibit adipogenesis and inflammatory response during adipocyte differentiation of 3T3-L1 cells.22 Brazilian red propolis (*Apis mellifera*), Copaifera oleoresins, flavonoid fraction of Bergamot Juice (*Citrus bergamia*), effusanin C (*Isodon japonicus*), oligomeric proanthocyanidins (*crataegus oxyacantha*) are others isolated compounds with anti-inflammatory actions in activated monocytes and macrophages (Montserrat-de la Paz et al., 2019). As well as prior studies (Millan-Linares et al., 2018, 2015), showing GPETAFLR as one of the major bioactive peptides with anti-inflammatory activity, our observations suggest that this octapeptide isolated from *Lupinus angustifolius L*. is associated with a remarkable prevention of oxidative and inflammatory in RPE cells. Therefore, dietary lupine protein intake could play a key role in maintaining ocular homeostasis.

VEGF, an important inducer of vascular permeability and a central regulator of new blood vessel growth, is remarkably involved in choroid neovascularization formation in wet AMD (Grisanti et al., 2015). In this process, it has been proposed that both oxidative and

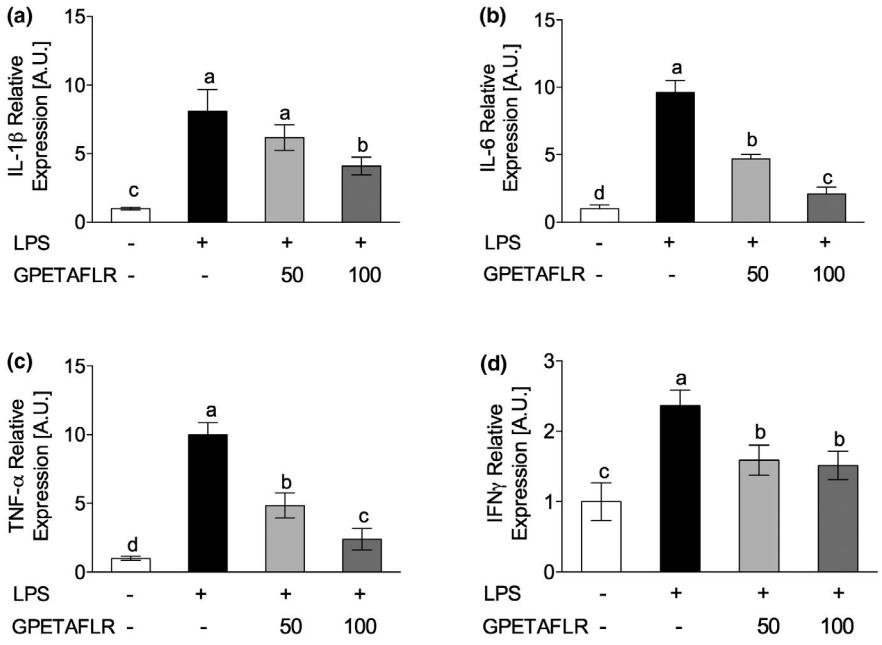

FIGURE 3 Gene expression of IL-1β (a), IL-6 (b), TNF-α (c), and IFNγ (d) relative to untreated cells (control) after the treatment of ARPE-19 cells with GPETAFLR at 50 and 100 μg/ml for 24 hr. Values are presented as means ± *SD* ($n = 3$). Different letters denote statistical differences ($p < .05$)

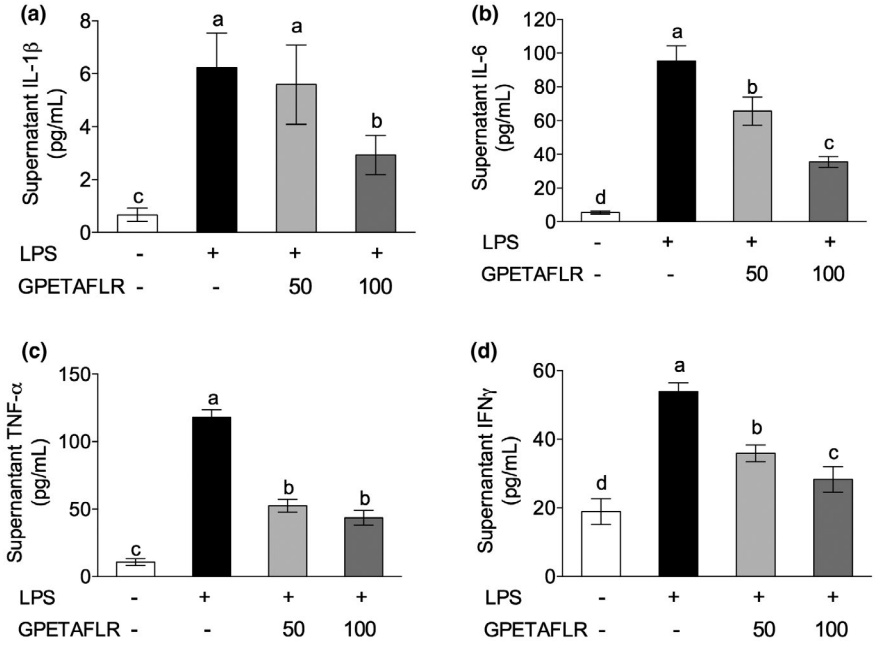

FIGURE 4 Cytokine release of IL-1β (a), IL-6 (b), TNF-α (c), and IFNγ (d) after the treatment of ARPE-19 cells with GPETAFLR at 50 and 100 μg/ml for 24 hr. Values are presented as means ± *SD* (*n* = 3). Different letters denote statistical differences ($p < .05$)

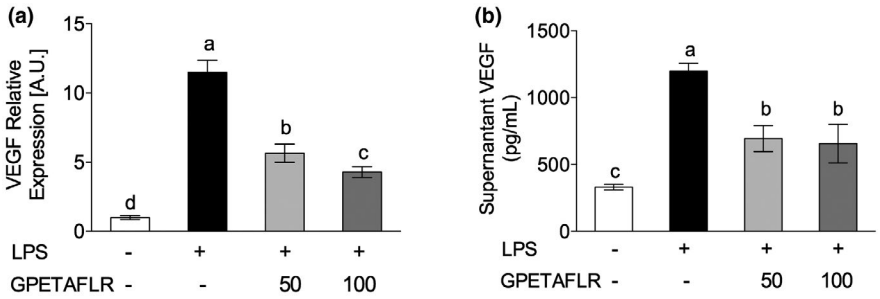

FIGURE 5 VEGF gene expression (a) and secretion (b) relative to untreated cells (control) after the treatment of ARPE-19 cells with GPETAFLR at 50 and 100 μg/ml for 24 hr. Values are presented as means ± *SD* (*n* = 3). Different letters denote statistical differences ($p < .05$)

inflammatory state of RPE may be also implicated (Fang, Yang, & Yang, 2014). Therefore, we investigated in ARPE-19 cells the effects of GPETAFLR on VEGF secretion and gene expression (Figure 5). Interestingly, the gene expression and protein secretion of VEGF was significantly reduced in human RPE cells exposed to GPETAFLR.

4 | CONCLUSIONS

We reveal a novel nutraceutical impact of GPETAFLR peptide in human RPE cells to prevent inflammatory cytokines, NO, GSH, and ROS. Briefly, our results support that the intake of *Lupine angustifolius* L., proposed to be a reservoir of GPETAFLR, could lessen the functional decay of RPE cells, leading therefore to a slowdown of the progress of AMD during age. Not only this work, but also future simple clinical studies should raise new nutritional strategies focused on understanding the etiological role of the foods, nutrition, and metabolism in the pathogenesis of ocular disorders.

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CONFLICTS OF INTEREST

The authors state no conflict of interest.

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