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

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Short title: GPETAFLR in RPE cells

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 AMD (age-related macular degeneration). In comparison with stimulated control cells, the peptide diminished the secretion and gene expression of VEFG, IL-1β, IL-6, IFNγ, and TNF-α, as well as ROS, GSH 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 APPLICATION

Dietary proteins are being investigated both from a functional or nutritional approach, as well as a raw material for peptides, since every source of dietary protein is susceptible to provide functional peptides. Bioactive plant peptide is a small amino acid sequence that has an active function as anti-inflammatory or antioxidative activity, among others. GPETAFLR is an octapeptide isolated from *Lupinus angustifolius* L. protein hydrolysates. This study concluded that peptide GPETAFLR-has a remarkable potential preventing inflammatory-related diseases. On the other hand, the consumption of *Lupinus angustifolius* L. appears to be helpful in the prevention and treatment of AMD.

Keywords: *Lupinus angustifolius*, Lupine seeds, Biopeptide, Retinal pigment epithelium, Age-related macular degeneration.

1 INTRODUCTION

Proteins from plants have raised as an option for the isolation of bioactive peptides (Lee & Hur, 2017; Pihlanto, Mattila, Makinen, & Pajari, 2017), short amino acid sequences which are inactive in the native protein. After being released by the action of digestive enzymes, these peptides can enter systemic circulation as bioactive molecules (Millan-Linares, Millan, Pedroche, & Yust, 2015). Previous studies in our lab have revealed the presence of a novel octapeptide isolated 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 (The protein structure models were generated by the automated SWISS-MODEL homology modeling pipeline, (Bienert et al., 2017) and its anti-oxidative and anti-inflammatory properties were tested in a human myeloid lineage (Millan-Linares et al., 2018). GPETAFLR has shown 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, Millan, Pedroche, & Yust, 2015), monocyte-derived osteoclasts (Millan-Linares et al., 2018), and primary human monocytes (Montserrat-de la Paz et al., 2019).

Many factors, including chronic inflammation, oxidative stress, age or nutrition may influence retinal pigment epithelium (RPE) causing dysfunction and degeneration (Levy et al., 2015). This epithelium consists of pigmented cells situated between the retina and choroidin a monolayer pattern**,** involved in the maintenance of the visual function and then in any retinal degenerative process (Pang, Zhou, & Kuang, 2018). These cells are responsible of the transport of nutrients between the retina and choroid as well as the volume and the chemical composition regulation of the subretinal space.

Consequently, retinal pigment epithelium appears to be a blood-retinal barrier (BRB) that plays a key role in protecting both the health and the integrity of retina and choroid (Qin et al., 2017; Sonoda et al., 2009). The inflammatory and immune response of the retina may involve RPE cells, as described in previous studies (Montserrat-de la Paz et al., 2016). Irreversible damage to the photoreceptors may be caused by a deficiency in integrity and functionality of retinal pigment epithelium, leading to a loss of central vision, known as age-related macular degeneration (AMD) (Montserrat-de la Paz et al., 2017a). Not only is AMD the main cause of blindness, but also keeps on increasing its predominance together with the population greater life expectancy and changes in life style worldwide (Montserrat-de la Paz et al., 2016).

Our results show for the first time that the novel plant peptide GPETAFLR is able to protect against RPE oxidative stress and inflammation. Therefore**,** this study proposes that the consumption of Lupine (*Lupinus angustifolius* L.) regarding the presence of GPETAFLR, may decrease the onset and progression of AMD.

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 et al., 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 37ºC / 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% foetal 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.

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 10^5 ARPE-19 cells/well, the culture was incubated during 24 h 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 h at 37ºC (Montserrat-de la Paz et al., 2017b). 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 h and then with CellROX Green Reagent (5 µM) for 30 min. Cells were washed with PBS and fixed with 3.7% formaldehyde, and the fluorescence signal was analysed in a Fluoroskan Microplate Fluorometer (ThermoFisher Scientific) equipped with a 485/555 excitation/emission filter set. The auto-fluorescence 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 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 h. 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 h 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 NO (nitric oxide) 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 LPS *in vitro*, ARPE-19 cells were cultured in 24 well plates at a 10^5 cells/well density and then treated 24 h 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 h 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 1000 ng RNA (iScript, BioRad). 20 ng 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 analysed 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 et al., 2017c; 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 (ANOVA), following Tukey multiple comparisons test as *post hoc* test. Those *P* values fewer 0.05 were determined statistically significant.

3 RESULTS AND DISCUSSION

Treatment during 24 h 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, Millan, Pedroche, & Yust, 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 analyse the preventive role of peptide GPETAFLR on oxidative conditions. H_2O_2 remarkably increased both intracellular ROS (**Figure 2b**) and nitrite (**Figure 2d**), compared to non-stimulated cells**.** The ROS production induced in presence of GPETAFLR was significantly lower than H_2O_2 control. 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 down-regulated by GPETAFLR (**Figure 2e**). Glutathione (GSH) protects against oxidative damage in many tissues, including RPE (Sun et al., 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 100μg/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; Millan-Linares, Millan, Pedroche, & Yust, 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 in RPE cells with a remarkable prevention of inflammatory pathways. Therefore, lupine proteins may play a key role in maintaining ocular tissues healthy**.** If so, inflammation at RPE level may create pathological conditions that could lead to AMD.

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

CONFLICTS OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grant AGL2012-40247-C02-01 from the Spanish Ministry of Economy and Competitiveness. Authors thank Cell Biology Unit of Instituto de la Grasa for its assistance during the fulfilment of this work and Research Scientist Enrique Martínez Force for his help with the protein structure models. SMP acknowledges financial support from "V Own Research Plan" (University of Seville).

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 specie

RPE: Retinal pigment epithelium

TNF: Tumour necrosis factor

VEGF: Vascular endothelial growth factor

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Figure Legends

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 colour was used to side chain, green colour was used to amino group, and blue colour was used to carboxyl group.

Figure 2. 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 h. Values are presented as means ± SD (n = 3). Different letters denote statistical differences ($P < 0.05$).

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 h. Values are presented as means ± SD (n = 3). Different letters denote statistical differences ($P < 0.05$).

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 h. Values are presented as means ± SD (n = 3). Different letters denote statistical differences ($P < 0.05$).

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 h. Values are presented as means ± SD (n = 3). Different letters denote statistical differences($P<0.05$).

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 1

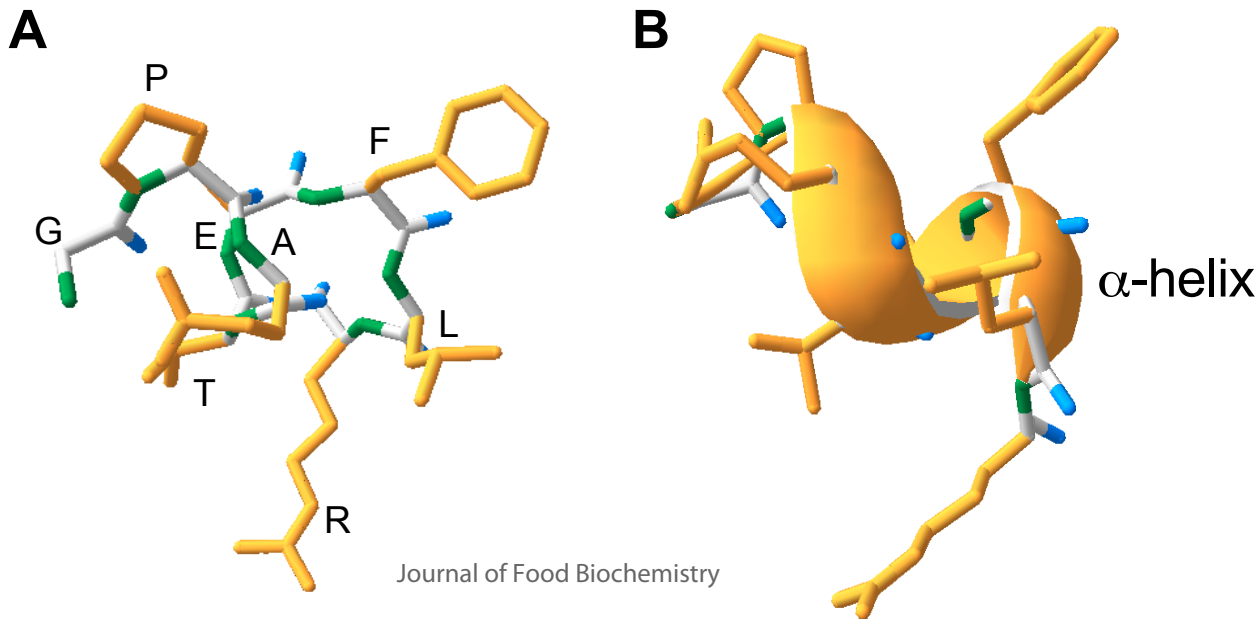

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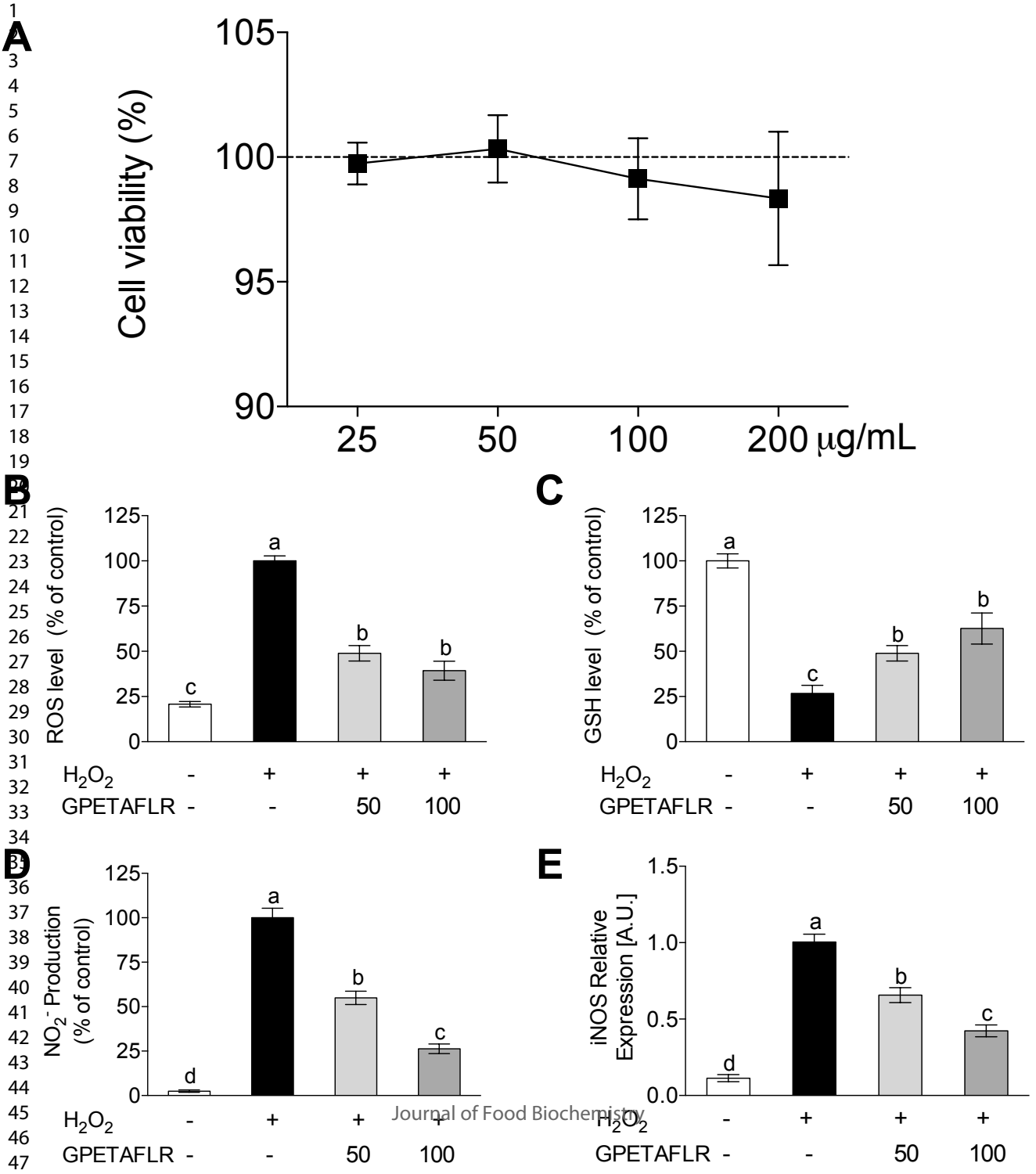
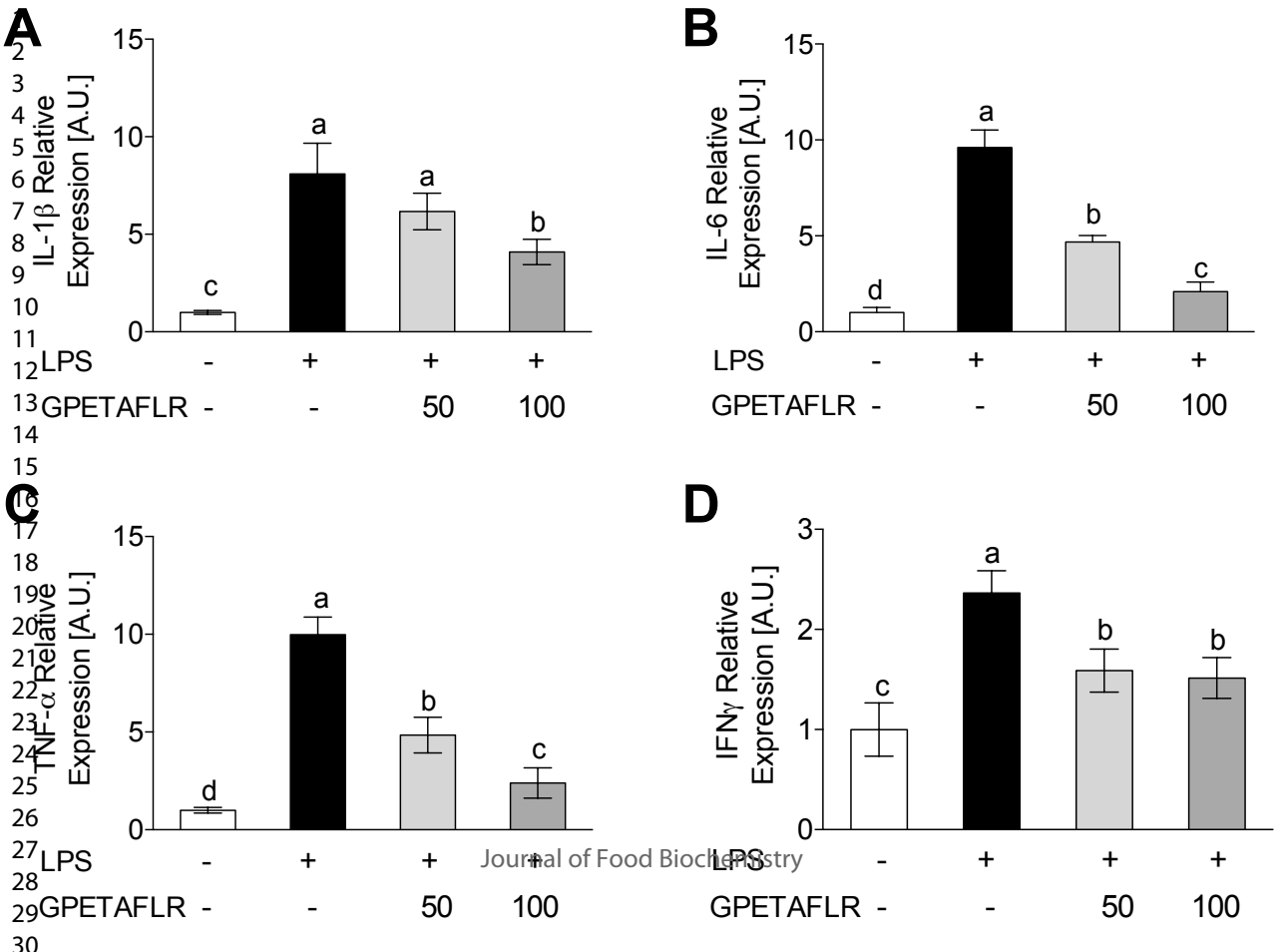
Figure 2





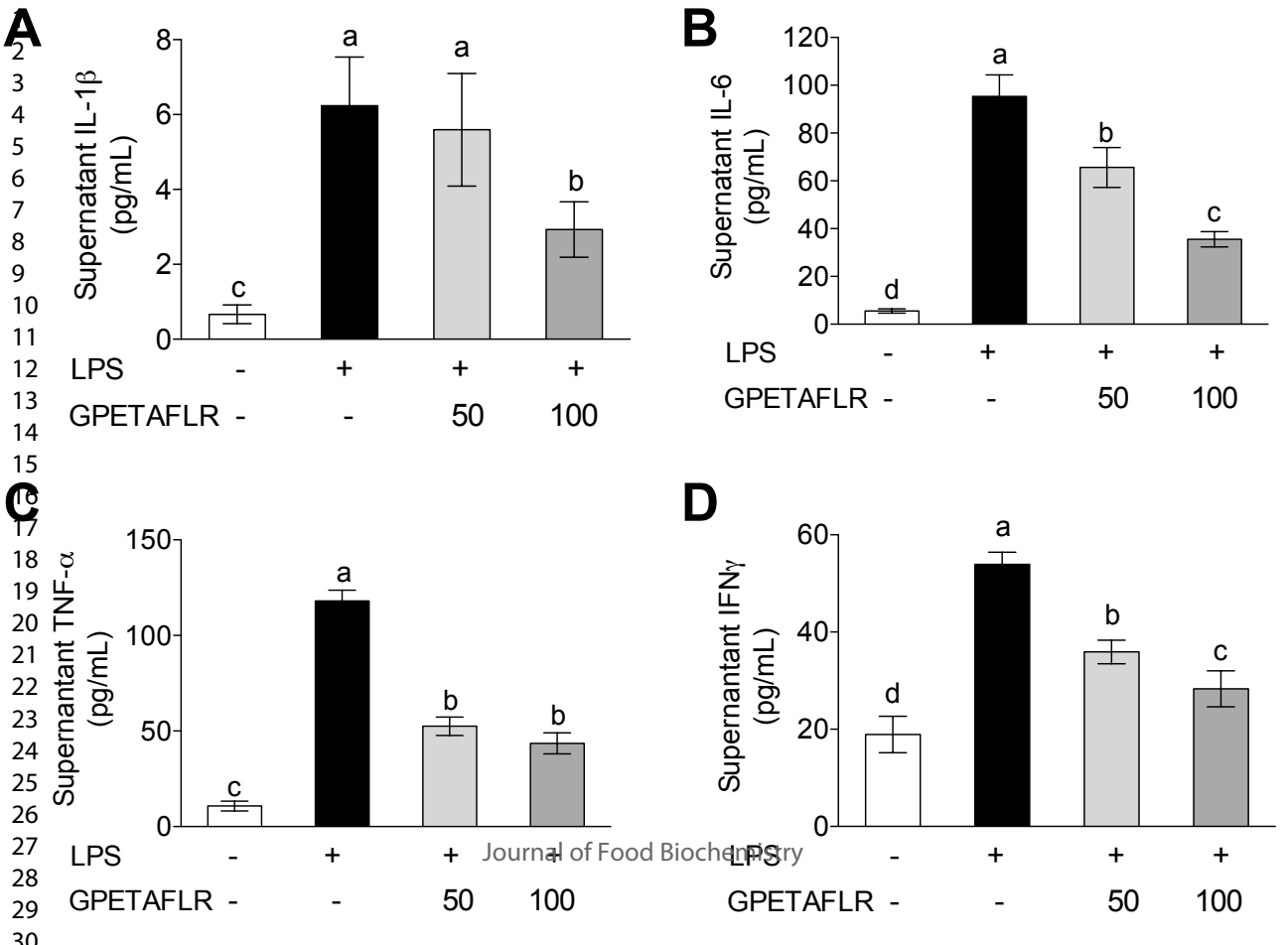


Figure 5