Minor compounds from virgin olive oil attenuate LPS-induced inflammation via visfatin-related gene modulation on primary human monocytes

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ABSTRACT

Our aim was to evaluate the effects of minor compounds found in the unsaponifibale fraction (UF) and in the phenolic fraction (PF) of virgin olive olive (VOO) on LPS-induced inflammatory response via visfatin modulation in human monocytes. For this purpose, monocytes were incubated with UF and PF at different concentrations and the pro-inflammatory stimulus LPS for 24 h; squalene (SQ) and hydroxytyrosol (HTyr), the main components in UF and PF respectively, were also used. The relative expression of pro-inflammatory and anti-inflammatory genes, and of genes related to the NAD+-biosynthetic pathway was assessed by RT-qPCR; and the secretion of some of these markers was analysed by ELISA. We found that UF, SQ, PF, and HTyr prevented from LPS-induced dysfunctional gene expression and secretion via visfatin-related gene modulation in human monocytes. These findings unveil a potential beneficial role for minor compounds of VOO in the prevention of inflammatory-disorders.

PRACTICAL APPLICATION

In this project potential health benefits of VOO micronutrients (Unsaponifiable and phenolic compounds) were confirmed through anti-inflammatory assays. These findings open new research lines for developing novel nutritional strategies with VOO as source of bioactive compounds to prevent the onset and progression of inflammation-related diseases.

Keywords: Olive oil, unsaponifiable, phenols, visfatin, NAMPT

1 INTRODUCTION

The consumption of extra virgin olive oil (VOO) appears to be a main determinant of the health benefits of Mediterranean diet (Montserrat-de la Paz et al., 2016a). Besides high levels of monounsaturated fatty acids (MUFAs), VOO contains a minor, but yet significant, amount of bioactive compounds such as phenolics and those found in the unsaponifiable matter (Lopez et al., 2014). Previous studies have demonstrated that both the phenolic fraction (PF) and unsaponifiable fraction (UF) from VOO positively influence oxidative stress and inflammation by modulating the expression of several genes involved in these processes (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, & Alarcon-de la Lastra, 2014; Cardeno, Sanchez-Hidalgo, & Alarcon-de la Lastra, 2013). Additionally, PF and UF from VOO have been shown to decrease cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) expression through nuclear factor kappa B (NF-κB)-mitogen-activated protein kinase (MAPK) signalling pathway in lipopolysaccharide (LPS)-activated murine macrophages (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, & Alarcon-de la Lastra, 2014). It has also been established that PF and UF from VOO exhibit anti-atherogenic *in vitro* and *in vivo* properties (Meza-Mirada et al., 2016; Montserrat-de la Paz et al., 2016b).

Nicotinamide phosphoribosyltransferase (NAMPT) or visfatin was originally reported as a pre–B-cell colony–enhancing factor secreted by activated leukocytes in bone marrow stroma (Samal et al., 1994). Intracellular NAMPT (iNAMPT) is the rate-limiting enzyme in the salvage pathway of nicotinamide adenine dinucleotide (NAD^+) generation (Montserrat-de la Paz, et al., 2017a). Accumulating evidence has highlighted the importance of NAMPT-mediated NAD^+ recycling, in concert with NAD^+-dependent protein deacetylases (sirtuins), on the expression of peroxisome proliferator–activated receptors (PPARs), NF-κB and endothelial NOS (eNOS), and

indirectly on cellular proliferation, differentiation, inflammation, aging and metabolism (Bermudez et al., 2017). NAMPT is also released by cells for giving rise to an extracellular pool of NAMPT (eNAMPT) that exerts cytokine-like activity (Dahl, Holm, Aukrust, & Halvorsen, 2012). We have previously shown increased expression of NAMPT within atherosclerotic carotid plaques (Dahl et al., 2007) and others and we have reported that eNAMPT promotes inflammatory and matrix degrading responses (Dahl, Holm, Aukrust, & Halvorsen, 2012). Therefore, to take advantage of the beneficial effects of minor compounds from VOO, the main aim of this study was to evaluate their ability to modulate LPS-mediated activation of primary human monocytes via visfatin/NAMPT-related gene modulation that are involved in the pathogenesis of numerous disorders, such as atherosclerosis.

2 MATERIAL AND METHODS

2.1 Preparation of UF and squalene (SQ) from VOO

EVOO of Picual variety was used as a matrix for UF extraction. UF was isolated following conventional procedures, as described IUPAC 2401 (Montserrat-de la Paz, Marin-Aguilar, Garcia- Gimenez, & Fernandez-Arche, 2014; Paquot, 1992). In brief, 5 g of VOO was saponified at 80 ºC by refluxing with 50 ml of 2 N potassium hydroxide solution in ethanol (Panreac, Barcelona, Spain), boiling gently until the solution became clear, and then for additional 20 min. Heating was stopped by addition of 50 mL distillate water through the top of the condenser and the solution was swirled. After cooling to 30-35 ºC, the solution was rinsed several times with water and UF was extracted with diethyl ether (Panreac) used as solvent for evaporation by distillation on a rotary evaporator (B-480 model, Büchi Labortechnik, Essen Germany) at 30 ºC under vacuum. Extracted material was dried in oven at 103

± 2 ºC for 15 min. Sample yields were between 1.3% and 1.4%. Quantitative analysis of UF aliphatic alcohols, sterols, and triterpenic dialcohols was performed according to European Regulation EEC/2568/91 for olive oil. Unsaponifiable matter was extracted as mentioned above and fractioned by thin layer chromatography (TLC) on a silica gel plate (Kieselgel 60 F254, Merck, Germany) with a mixture of hexane and ethylether (65:35 v/v). Aliphatic alcohols, sterols, and triterpenic dialcohols were identified by their retention factor. The triterpenic dialcohols, erythrodiol and uvaol, recovered from the plate were transformed into trimethylsilyl ethers by adding 200 µL of a mixture of 9:3:1v/v/v of pyridine-hexamethyldisilazane-trimethylchlorosilane and analysed by gas chromatography (GC) using an HP 5890 series II gas chromatograph equipped with a flame ionization detector and a 30 m, 0.32 mm i.d., Tracsil TRB-5 (95% dimethylpolysiloxane, 5% diphenyl, film thickness 0.25 µm) capillary column (Teknokroma, Barcelona, Spain). The chromatographic conditions were as follows: injector 300 ºC, isothermal column 275 ºC, and detector 300 ºC. The split ratio was 1:50 and the hydrogen flow rate of 1.0 ml/min, 130 Kpa. The chromatographic conditions for alcohol determinations were the same as those mentioned above for sterols and triterpene diols, except that oven temperature was 215 ºC (5 min) and then 3 ºC/min increased to 290 ºC, and held for 2 min. The quantifications were based on the peak areas identified by their relative retention time. The quantification of tocopherols was based on the comparison of the peak areas with those of an external standard curve of R-tocopherol and identified by high-performance liquid chromatography (HPLC) (Montserrat-de la Paz, Fernandez-Arche, Angel-Martin, &Garcia-Gimenez, 2014). The test sample was prepared as a solution to 10% by weight in hexane (Panreac) and analysed in an HPLC system (Hewlett–Packard, Minnesota, US 1050) equipped with a fluorescence detector (Shimadzu RF-535)

using an excitation wavelength set at 290 nm, an emission wavelength at 330 nm, and an HPLC analytical column silica (250 mm x 4 mm i.d. x 5 µm) (Merck, Superspher Si60) at a temperature of 30 ºC. A flow rate of 1 mL/min, 400 bar was used. Total concentrations were calculated as the sum of individual concentrations. Results were expressed as mg/100 mg UF (**Table 1**).

The quantification of squalene (SQ) was based on the comparison of the peak areas with those of an external standard curve and identified by HPLC. VOO (0.5 g) with 1 mL internal SQ standard (Sigma-Aldrich, St Louis, MO, USA) (5 mg/mL) were weighed and mixed; then, 2 mL of heptane and 400 mL of methanolic solution of potassium hydroxide 2 N were added and the mix was shaken vigorously. When the upper solution became clear, 1 µL was injected into a GC. GC analysis of SQ content was performed using an HP5890 (Hewlett-Packard) gas chromatograph fitted with a flame ionization detection system and a split injection system (split ratio 1:50). Separations were carried out on a fused silica capillary column SPB-5 (30 m x 0.25 mm i.d.) coated with 5% phenyl-95% methylsilicone, with a thickness of 0.25 µm (Supelco). The operating conditions were as follows: injector temperature, 280 ºC; detector temperature, 300 ºC; oven programming temperatures, initial 240 ºC for 6 min and then rising at 5 ºC/min to 280 ºC. A hydrogen flow rate of 1 mL/min, 130 Kpa was used. Total concentration was calculated as the sum of individual concentrations. Results were expressed as mg/100 mg UF.

2.2 Preparation of PF and hydroxytyrosol (HTyr) from VOO

PF was obtained using the method described by Vazquez-Roncero *et al.* (Vazquez-Roncero, Janer del Valle, & Janer del Valle, 1973) with some modifications. An amount of 50 g of VOO was extracted with 125 mL of methanol:water (80:20, v/v).

The resultant was mixed with a vortex at 5000 *g* for 1 min and then sonicated for 15 min. After decantation, the methanolic phase was concentrated in vacuum under a stream of nitrogen at a temperature lower than 35 ºC until it reached a syrupy consistency. PF was lyophilized and stored at –80 ºC until its use. Quantitative and qualitative analysis of PF was performed according to the COI/T20/29. The method is based on direct extraction of the phenolic minor polar compounds by means of a methanol solution and subsequent quantification by HPLC. After direct extraction, an aliquot of the supernatant phase was taken, filtered, and injected into a HPLC system equipped with C18 reverse-phase column (25 cm x 4.6 mm), type Spherisorb ODS-2 (5 mm) and a spectrophotometric UV detector at 280 nm. The content of phenols was expressed in mg of tyrosol per kg of oil and was calculated by measuring the areas of the related chromatographic peaks. The composition of the isolated PF is detailed in **Table 2**. To *in vitro* treatments, both HTyr and SQ were purchased by Sigma-Aldrich (Madrid, Spain).

2.3 Blood collection and isolation of human monocytes

This study was conducted according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Informed consent for the study was obtained from healthy male blood donors (age <35 years) at the University Hospital Virgen del Rocio, Seville. Participants declared that they were non-smokers and were not taking any medication. Peripheral blood samples were drawn from a large antecubital vein and collected into K3EDTA-containing tubes (Becton Dickinson, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples by centrifugation over a Ficoll-Histopaque (Sigma) gradient. Monocytes were isolated

from PBMCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain) according to the manufacturer's instructions. The purity for CD14 monocyte isolations was routinely >90% by flow cytometry (FACScanto II flow cytometer and FACSDiva software, BD). Following isolation, monocytes were suspended in a RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated foetal bovine serum.

2.4 Cell treatments

Monocytes (5 x 10^5 cells/mL) were incubated with LPS (100 ng/ml) in the absence or presence of UF at 25 and 50 µg/ml, SQ at 50 µM, PF at 25 and 50 µg/ml, or HTyr at 41 µM from VOO for 24 h. Untreated cells were considered as negative control, whereas those treated with only LPS were considered as positive control. After the treatments in 24-well plates, supernatants were removed, and stored at -20 ºC until use. Cells were used to RNA extraction.

2.5 RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted by using Trisure Reagent (Bioline). RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). RNA (1 µg) was subjected to reverse transcription (iScript, BioRad). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (BioRad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (BioRad) containing the primer pairs for the corresponding gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping genes. The sequence and information for the primers are shown in

ESI Table 1. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (GAPDH) gene content and expressed as percentage of controls.

2.6 Measurement of cytokine release

The levels of visfatin, IL-1β, IL-6, TNF-α, and IL-10 in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA), following the indications of the manufacturer (Diaclone, Besancon, France). The cytokine concentrations were expressed in pg per mL, as calculated from the calibration curves from serial dilution of human recombinant standards in each assay.

2.7 Statistical analysis

All values are expressed as arithmetic means ± standard deviations (SD). Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as *post hoc* test. *P* values less than 0.05 were considered statistically significant.

3 RESULTS

3.1 Effects of minor compounds from VOO on visfatin/eNAMPT secretion in LPS-treated human monocytes

The effects of UF, SQ, PF and HTyr of VOO on secretion of visfatin (eNAMPT) in human primary monocytes are showed in **Figure 1.** LPS induced an increase in the concentration of visfatin (eNAMPT) in the medium of human primary monocytes compared to untreated control cells. Interestingly, this LPS-induced secretion of visfatin was prevented by UF at 50 μg/mL, PF at 25 and 50 μg/mL and HTyr at 50 μM but not by UF at 25 μg/mL or SQ at 41 μM. In the case of PF, the effect was dependent on concentration (50 μg/mL > 25 μg/mL, $P < 0.05$). These observations suggest that minor compounds in VOO, probably phenolics such as HTyr, may have a beneficial role in regulating visfatin secretion in activated human monocytes.

3.2 Effects of minor compounds from VOO on expression of NAMPT- and inflammatory-related genes in LPS-treated human monocytes

Genes involved in the NAD^+ biosynthetic salvage pathway, including NAMPT (**Figure 2A**), NMNAT-1 (**Figure 2B**) and NMNAT-3 (**Figure 2D**), were downregulated by LPS in human primary monocytes; however, NMNAT-2 gene was unaffected (**Figure 2C**). When LPS-treated cells were incubated in the presence of UF, PF, HTyr and SQ not only the effect of LPS on the above genes was abrogated (e.g. NAMPT gene with UF at 25 μg/mL and 50 μg/mL and SQ at 50 μM) but also their transcriptional activity was upregulated. This was particularly prominent with PF and HTyr, and concentration-dependent (50 μg/mL > 25 μg/mL, $P < 0.05$) with PF on NMNAT-1 and NMNAT-2 genes. In addition, the expression of genes encoding NAD^+-dependent deacetylase SIRT-1 (**Figure 2E**) and its downstream target PPARγ

(**Figure 2F**) was repressed by LPS but upregulated in the presence of VOO-derived fractions or compounds, with notable effects of PF and HTyr and with the exception of SQ that only retained mRNA of PPARγ gene to a level similar to that of untreated control cells. We also observed that LPS-induced upregulation of MCP- 1 gene (which encodes an important factor for the recruitment of cells within foci of inflammation) and CCR2 gene (which encodes the receptor for MCP-1) was abolished by either UF, PF, HTyr or SQ, reaching mRNA levels lower than those found in the untreated control cells by PF at 50 μg/mL or HTyr at 41 μM. These observations suggest that minor compounds in VOO, probably SQ and phenolics such as HTyr, may have a beneficial role in regulating NAD^+ biosynthetic pathway and the inflammatory response in activated human monocytes.

3.3 Effects of minor compounds from VOO on gene expression and of pro- and anti-inflammatory cytokines in LPS-treated human monocytes

To gain insight into the effects of VOO-derived fractions and compounds in human primary monocytes upon LPS treatment, we analysed secretion and gene expression of pro-inflammatory (IL-1β, IL-6, and TNF-α) and anti-inflammatory (IL-10) cytokines. UF, PF, HTyr and SQ diminished LPS-induced release of IL-1β (**Figure 3A**), IL-6 (**Figure 3B**) and TNF-α (**Figure 3C**) as well as expression of genes encoding these cytokines (**Figures 3E-3G**). In the case of UF and PF, their effects were concentration-dependent (50 μg/mL > 25 μg/mL, $P < 0.05$). Secretion of anti-inflammatory IL-10 was increased by UF at 50 μg/mL, PF at 25 and 50 μg/mL and HTyr at 50 μM (**Figure 3D**) while all tested VOO-derived fractions and compounds increased the transcriptional activity of IL-10 gene (**Figure 3H**) above the level of untreated control cells. The effects of UF and PF on IL-10 secretion and of PF on IL-

10 gene expression were concentration-dependent (50 μg/mL > 25 μg/mL, $P < 0.05$). These observations reinforce the notion that minor compounds of VOO, probably SQ and phenolics such as HTyr, may have a role in reducing the inflammatory response in activated human monocytes.

4 DISCUSSION

The literature often refers to "Mediterranean diet" as protective due to the prevention of cardiovascular disease by improving classical risk factors and by promoting an intense anti-inflammatory effect, and "meat-based" or "Westernized" diets as inductive of an inflammatory state (Naranjo et al., 2016). One of the key processes of inflammation is the activation of circulating leukocytes (Montserrat-de la Paz et al., 2017b). Among the leukocytes, monocytes are the first immune cells that respond quickly to injury and its activation causes the increase and perpetuation of the inflammatory state (Libby, Nahrendorf, & Swirski, 2016). Herein, we aimed to address the relevance of UF from VOO in modulating LPS-mediated inflammatory response in human primary monocytes. Our study shows that UF and PF, as well as HTyr from VOO could reduce the inflammatory response via downregulation of visfatin. This protein has a potential involvement in inflammation and in a wide range of other disorders, including myocardial failure, atherosclerosis, metabolic syndrome, malignancies, neurodegeneration, and aging (Montserrat-de la Paz et al., 2014). There is recent evidence of LPS targeting visfatin as a biomarker of inflammation (Iwasa et al., 2016). But the role of visfatin/NAMPT is controversial. Recent studies have been focused on eNAMPT, which is thought to act as a pro-inflammatory cytokine (Laiguillon et al., 2014). However, *Lin et al.* (2015) and our research group also reported that iNAMPT may have beneficial anti-inflammatory properties (Bermudez

et al., 2017). This dual role of eNAMPT (visfatin) and iNAMPT has been explored in the present study by analyzing the concentration of visfatin/eNAMPT in the medium of cells and the relative mRNA levels of NAMPT/iNAMPT. Other compounds in the unsaponifiable of olives, such as oleanolic acid and related triterpenoids, have been shown to inhibit visfatin secretion in murine 3T3-L1 adipocytes (Rodriguez-Rodriguez, 2015). The effects on visfatin secretion that we observed were dependent on the concentration of UF and PF from VOO, suggesting that these fractions, probably due to HTyr, have anti-inflammatory properties through regulation of visfatin exit from cells. Our findings are also in line with a previous study in which UF of VOO exhibited antioxidant and inhibitory activity of NF-κB-mediated signalling in murine peritoneal macrophages treated with LPS (Cardeno, Sanchez-Hidalgo, Aparicio-Soto, & Alarcon-de la Lastra, 2014). It is noteworthy that a nutritional intervention demonstrated that the ingestion with VOO (25 mL/day, 577 mg of phenolic compounds/kg) for 4 weeks reduced serum levels of visfatin/eNAMPT in 30 patients with type-2 diabetes (Santangelo et al., 2016). This effect was accompanied with an improvement in the metabolic control of glucose and in the profile of circulating inflammatory adipokines.

Our study is the first in showing a role for UF, PF, SQ, and HTyr from virgin olive oil in the regulation of genes involved in NAD^+ biosynthesis, such as NAMPT (iNAMPT), NMNAT-1, NMNAT-2, and NMNAT-3. NMNAT-1 has been recently reported to exhibit protection of neuronal function against tau-induced inflammation and atrophy in a mouse model of Alzheimer´s disease (Musiek et al., 2016). The potential role of NMNAT family members in the biology of innate immune cells and in the inflammatory response mediated by monocytes remains to be established. The fact that LPS downregulated the gene encoding NAMPT suggests that iNAMPT is not

involved in the inflammatory response induced by LPS in human monocytes. These findings could be indicative of contrary activities of visfatin/eNAMPT and iNAMPT in inflamed tissues (Benito-Marin et al., 2014).

Because NAMPT is a key NAD^+ biosynthetic enzyme and SIRT1 requires NAD^+ for its enzymatic activity that includes the abolishment of inflammatory mediator production and cell recruitment (Busch, Mobasheri, Shayan, Stahlmann, & Shakibaei, 2012; Dong et al., 2014), we also analysed the impact of the UF, PF, SQ, and HTyr from VOO in the gene regulation of SIRT1 and its downstream target PPARγ (Qu et al., 2016). In a recent study, SIRT1 has been described as a critical immune regulator whose expression can be downregulated in human macrophages following the release of inflammatory cytokines upon cell stimulation with LPS (Jia et al., 2016). In contrast, the deficiency of SIRT1 in human monocytes and mononuclear cells has been associated to systemic and possibly vascular wall inflammation, leading to atherogenesis (Kitada, Ogura, & Koya, 2016). In human monocytes, we found that all tested fractions or compounds of VOO reversed the reduction of SIRT1 gene expression caused by LPS. These findings are similar to those reported with the polyphenol resveratrol in TNF-α treated human endothelial cells (Liu et al., 2017). Furthermore, we observed that the gene expression profile for PPARγ was similar as to SIRT1; the UF, PF, and HTyr not only restoring but also potentiating PPARγ gene expression in LPS-treated human monocytes. PPARγ agonists were reported to suppress inflammation by inhibiting NF-κB signalling pathways in endothelial cells (Marcone, Haughton, Simpson, Belton, & Fitzgerald, 2015). Therefore, our study suggests that compounds in the UF of VOO may counteract LPS-induced pro-inflammatory response by restoring mRNA levels of SIRT1 and PPARγ genes in human monocytes.

To gain more insight into the anti-inflammatory action of UF, PF, SQ, and HTyr from VOO in human monocytes, we investigated whether these fractions or compounds played a role in the regulation of MCP-1 and CCR2. MCP-1, also known as CCL2, is mainly produced by monocytes/macrophages in response to several stimuli and conditions such as cytokines, oxidative stress or growth factors (Deshmane, Kremlev, Amini, & Sawaya, 2009). The binding of this chemokine to the receptor CCR2 facilitates the process of both migration and infiltration of several cell systems such as monocytes, natural killer cells, T lymphocytes, and memory cells (Vakilian, Khorramdelazad, Heidari, Rezaei, & Hassanshahi, 2017). We found that all tested fractions or compounds of VOO avoided LPS-induced up-regulation of MCP-1 and CCR2 genes in human monocytes. Other natural polyphenols such as curcumin have been shown to exert similar effects on MCP-1 gene expression in several cell lines (Karimian, Pirro, Majeed, & Sahebkar, 2016). Our findings were consistent with the notion that UF and PF, and notably HTyr of VOO may be useful for preventing recruitment and chemotaxis of monocytes across the vascular endothelium.

These observations were consistent with the decrease in the medium and prevention of LPS-induced gene expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in human monocytes. All the fractions (in a dose-dependent manner), SQ, and HTyr exerted these beneficial effects. Of note, they also promoted the release and gene expression of the anti-inflammatory cytokine IL-10. Our study is complementary to a previous one in which squalene was shown to reduce mRNA levels of pro-inflammatory cytokines in human monocytes and neutrophils (Cardeno et al., 2015). This is in line with evidence of anti-inflammatory properties of UF of virgin olive oil (Amiot, 2014; Cardoso, Catarino, Semiao, & Pereira, 2014).

5 CONCLUSIONS

This study unveils new beneficial effects of UF, including SQ, PF, and HTyr, from VOO and establishes the potential use of these fractions/compounds in the prevention and treatment of inflammatory disorders.

Abbreviations

AMPK: AMP-activated protein kinase

COX: Cyclooxygenase

CCR2: Chemokine receptor type 2

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HTyr: Hydroxytyrosol

LPS: Lipopolysaccharide

MCP-1: Monocyte chemoattractant protein-1

MUFA: Monounsaturated fatty acid

NAD: Nicotinamide adenine dinucleotide

NAMPT: Nicotinamide phosphoribosyltransferase

NF-κB: Nuclear factor-kappa B

NMNAT: Nicotinamide mononucleotide adenyltransferase

NOS: Nitric oxide synthase

PPARγ: Peroxisome proliferator-activated receptor gamma

SIRT: Sirtuin

SQ: Squalene

TNF-α: Tumour necrosis factor α

UF: Unsaponifiable fraction

CONFLICTS OF INTEREST

The authors state no conflict of interest.

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Figure Legends

Figure 1. Effects of UF, SQ, PF, and HTyr from VOO on visfatin/eNAMPT secretion in LPS-treated human monocytes. Cells were untreated (C) or treated with LPS (100 ng/ml) in the absence (LPS) or presence of UF (at 25 and 50 μg/ml), SQ (at 50 μM), PF (at 25 and 50 μg/ml) or HTyr (at 41 μM) for 24 h. The concentration of visfatin in culture supernatants was measured by ELISA. Values shown are the mean ± SD (n = 3) and those marked with different lowercase letters are significantly different ($P < 0.05$).

Figure 2. Effects of UF, SQ, PF, and HTyr from VOO on expression of NAMPT- and inflammatory-related genes in LPS-treated human monocytes. Cells were untreated (C) or treated with LPS (100 ng/ml) in the absence (LPS) or presence of UF (at 25 and 50 μg/ml), SQ (at 50 μM), PF (at 25 and 50 μg/ml) or HTyr (at 41 μM) for 24 h. The expression of NAMPT (A), NMNAT-1 (B), NMNAT-2 (C), NMNAT-3 (D), SIRT-1 (E), PPARγ (F), MCP-1 (G), and CCR2 (H) genes was measured by qPCR. Values shown are the mean ± SD (n = 3) and those marked with different lowercase letters are significantly different ($P < 0.05$).

Figure 3. Effects of UF, SQ, PF, and HTyr from VOO on secretion and gene expression of pro- and anti-inflammatory cytokines in LPS-treated human monocytes. Cells were untreated (C) or treated with LPS (100 ng/ml) in the absence (LPS) or presence of UF (at 25 and 50 μg/ml), SQ (at 50 μM), PF (at 25 and 50 μg/ml) or HTyr (at 41 μM) for 24 h. The concentration of IL-1β (A), IL-6 (B), TNF-α (C), and IL-10 (D) in culture supernatants was measured by ELISA, whereas the expression of IL- 1β (E), IL-6 (F), TNF-α (G), and IL-10 (H) genes was measured by

qPCR. Values shown are the mean ± SD (n = 3) and those marked with different lowercase letters are significantly different ($P < 0.05$).

Table 1. Main composition of UF from EVOO.

Component	mg/Kg (ppm)
Squalene	5681
Total sterols	1289
Campesterol	40.7
β-sitosterol	7.8
Δ5-Avenasterol	69.7
Total methylsterols	69.8
Total triterpenic alcohols	568
Total aliphatic alcohols	163
C24	40.8
C26	71.9
α-tocopherol	87

Table 2. Main composition of PF from EVOO.

Component	µM Phenol (50 µg PF/mL)
Hydroxytyrosol	41.07
Tyrosol	43.09
Pinoresinol	6.21
Cinnamic acid	6.89
Oleuropein aglycone	39.18
Luteolin	4.18

ESI Table 1. Sequences of RT-qPCR primers for gene expression analysis.

Target	No. *GenBank*	Direction	Secuence (5' --> 3')
Visfatin/NAMPT	NM_005746.2	Forward	GCCAGCAGGGAATTTTGTTA
		Reverse	TGATGTGCTGCTTCCAGTTC
NMNAT-1	NR_022787.3	Forward	TCTCCTTGCTTGTGGTTCATTC
		Reverse	TGACAACTGTGTACCTTCCTGT
NMNAT-2	NM_015039.3	Forward	GTAAAACGACGGCCAGT
		Reverse	TAATACGACTCACTATAGG
NMNAT-3	NM_001200047.2	Forward	GTAAAACGACGGCCAGT
		Reverse	TAATACGACTCACTATAGG
SIRT-1	NM_012238.4	Forward	CAGACCCTCAAGCCATGTTT
		Reverse	TAATACGACTCACTATAGG
PPARγ	NM_001330615.1	Forward	CTGAATGTGAAGCCCATTGAA
		Reverse	GATCCTTTGGATTCCTGAAA
MCP-1	NM_011333.3	Forward	ACTGAAGCTCGTACTCTC
		Reverse	CTTGGGTTGTGGAGTGAG
CCR2	NM_001123041.2	Forward	AGAGGCATAGGGCAGTGAGA
		Reverse	GCAGTGAGTCATCCCAAGAG
TNF-α	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
IL-1β	NM_000576	Forward	GGGCCTCAAGGAAAAGAATC
		Reverse	TTCTGCTTGAGAGGTGCTGA
IL_6	NM_000600	Forward	TACCCCCAGGAGAAGATTCC
		Reverse	TTTTCTGCCAGTGCCTCTTT
IL-10	NM_001289746	Forward	ATGCACAGCTCAGCACTGC
		Reverse	GTTTCGTATCTTCATTGTCATGTAGG

GAPDH	NM_002982.3	Forward	CACATGGCCTCCAAGGAGTAAG
		Reverse	CCAGCAGTGAGGGTCTCTCT

Figure 1

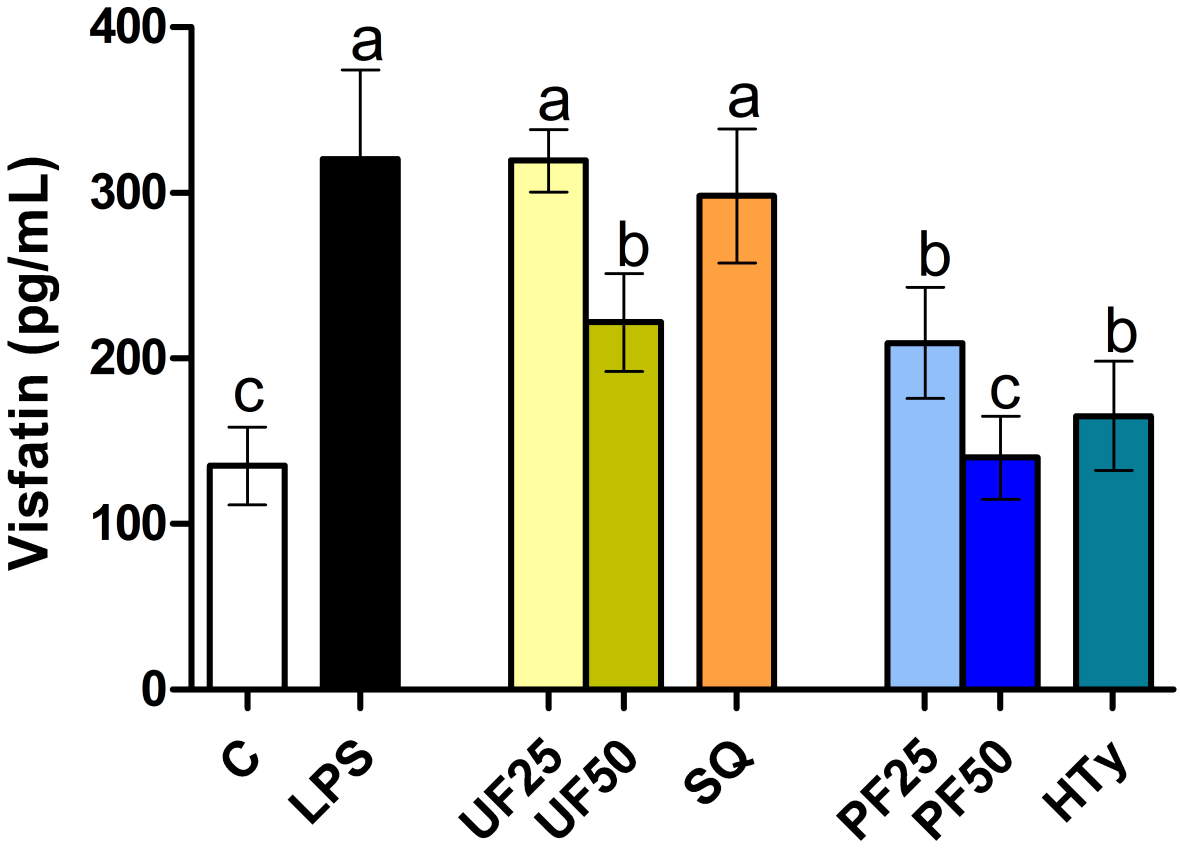

Visfatin (pg/mL)
400
300
200
100
0
a
a
a
b
b
c
c
b
C
LPS
UF25
UF50
SQ
PF25
PF50
HTy

Figure 2

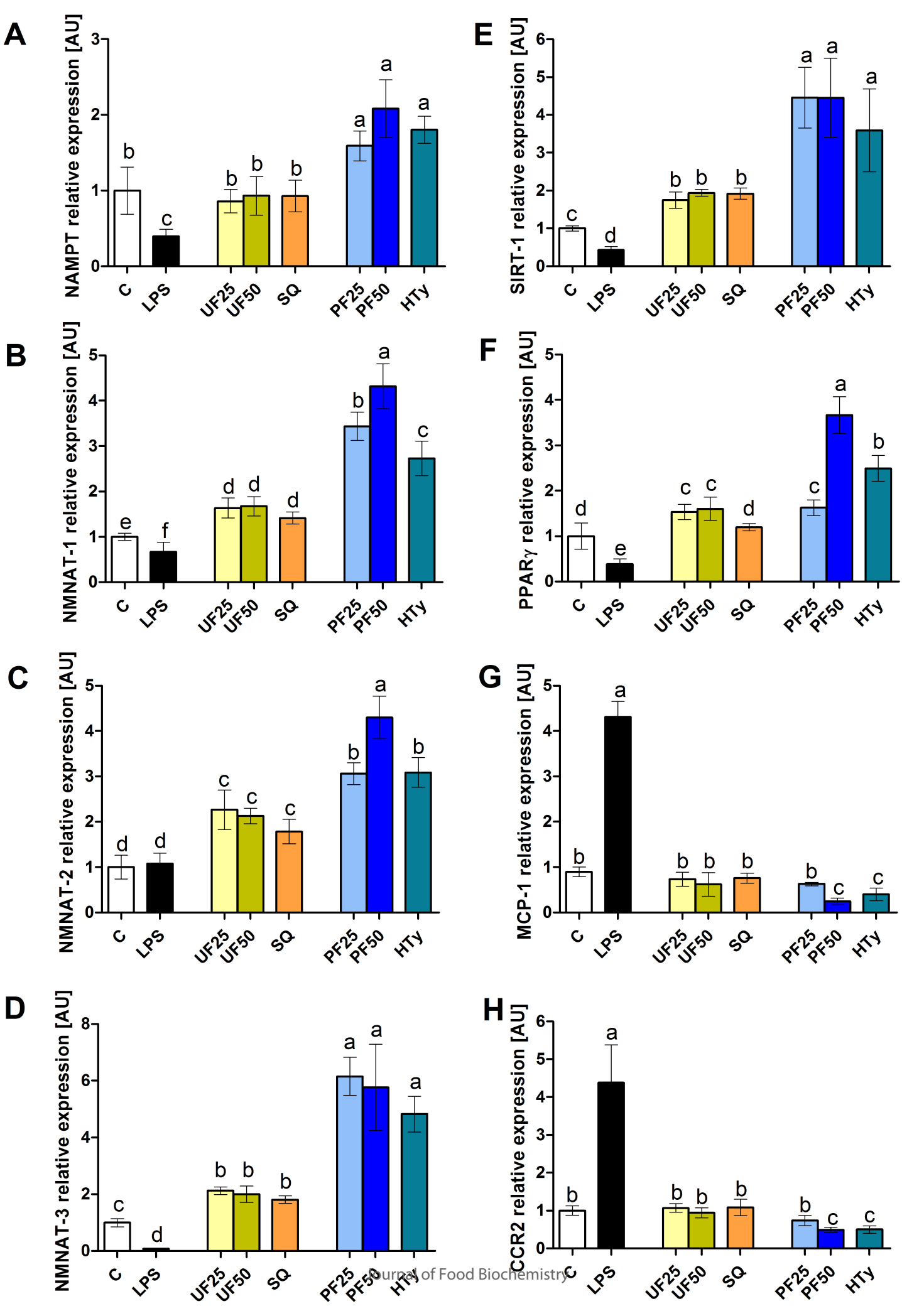
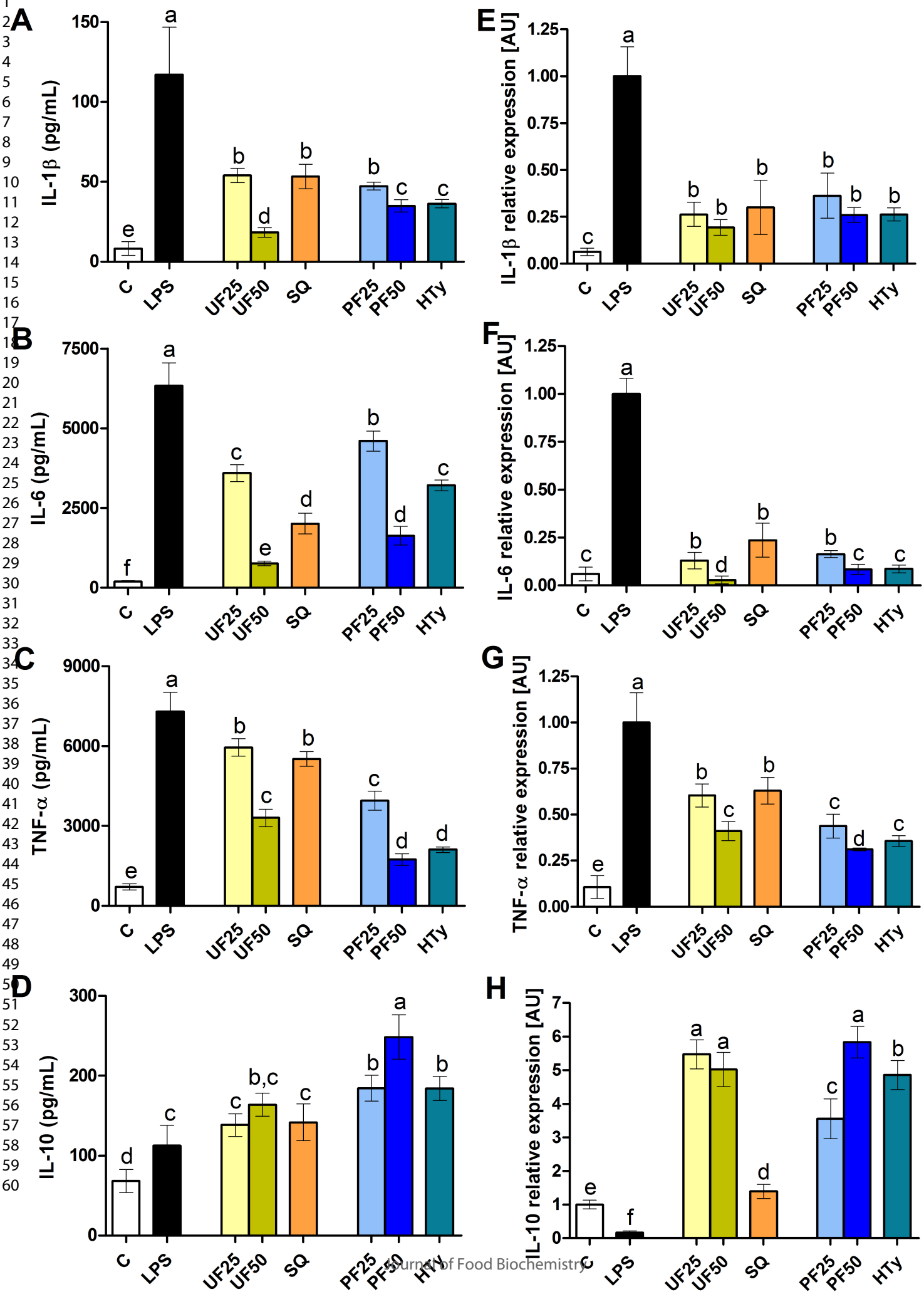

A
NAMPT relative expression [AU]
B
NMNAT-1 relative expression [AU]
C
NMNAT-2 relative expression [AU]
D
NMNAT-3 relative expression [AU]
E
SIRT-1 relative expression [AU]
F
PPARγ relative expression [AU]
G
MCP-1 relative expression [AU]
H
CCR2 relative expression [AU]
C
LPS
UF25
UF50
SQ
PF25
PF50
HTy

Figure 3


A
IL-1β (pg/mL)
150
100
50
0
e
a
b
d
b
b
c
c
C
LPS
UF25
UF50
SQ
PF25
PF50
HTy
E
IL-1β relative expression [AU]
1.25
1.00
0.75
0.50
0.25
0.00
c
a
b
b
b
b
b
B
IL-6 (pg/mL)
7500
5000
2500
0
f
a
c
e
d
b
d
c
F
IL-6 relative expression [AU]
c
a
b
d
b
b
c
c
C
TNF-α (pg/mL)
9000
6000
3000
0
e
a
b
c
b
c
d
d
G
TNF-α relative expression [AU]
e
a
b
c
b
c
d
c
D
IL-10 (pg/mL)
300
200
100
0
d
c
c
b,c
c
b
a
b
H
IL-10 relative expression [AU]
7
6
5
4
3
2
1
0
e
f
a
a
d
c
a
b