

Obtaining from Grape Pomace an Enzymatic Extract with Anti-inflammatory Properties

B. Rodríguez-Morgado · M. Candiracci ·
C. Santa-María · E. Revilla · B. Gordillo ·
J. Parrado · A. Castaño

Abstract Grape pomace, a winemaking industry by-product, is a rich source of bioactive dietary compounds. Using proteases we have developed an enzymatic process for obtaining a water-soluble extract (GP-EE) that contains biomolecules such as peptides, carbohydrates, lipids and polyphenols in soluble form. Of especial interest is its high polyphenol content (12 %), of which 77 % are flavonoids and 33 % are phenolic acids. The present study evaluates *in vitro* the potential anti-inflammatory effect of GP-EE by monitoring the expression of inflammatory molecules on N13 microglia cells stimulated with lipopolysaccharide (LPS). GP-EE decreases the mRNA levels of the inflammatory molecules studied. The molecules under study were as follows: inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), the ionized calcium binding adaptor molecule-1(Iba-1) and the Toll like receptor-4 (TLR-4), as well as the iNOS protein level in LPS-stimulated microglia. Our findings suggest that, as a result of its ability to regulate excessive microglial activation, GP-EE possesses anti-inflammatory properties. Therefore, acting as a chemopreventive agent, it may be of therapeutic interest in neurodegenerative diseases involving neuroinflammation. We can,

therefore, propose GP-EE as a useful natural extract and one that would be beneficial to apply in the field of functional foods.

Keywords Grape pomace · Enzymatic extract · Polyphenols · Anti-inflammatory · Neurodegeneration

Abbreviations

GP	Grape pomace
GP-EE	Grape pomace enzymatic extract
LPS	Lipopolysaccharide
iNOS	Inducible nitric oxide synthase
TNF- α	Tumor necrosis factor- alpha
IL-1 β	Interleukin-1 β
TLR-4	Toll like receptor-4
Iba-1	Ionized calcium binding adaptor molecule-1
UPLC	Ultra-high performance liquid chromatography
RT	Reverse transcription

Introduction

Grape pomace, a winemaking industry by-product, has traditionally been considered as an environmental problem. However, GP is being increasingly recognized as a source of bioactive dietary compounds such as polyphenols [1]. To date, its efficient recovery to convert it into a high value-added product has been accomplished by conventional solid–liquid extraction with organic solvents (ethanol, methanol, ethyl ether, ethyl acetate, *etc.*) or by heated sulphurated water. It is also recovered enzymatically with carbohydrate hydrolases (cellulase and pectinase [1–5] and by physical methods such as superheated liquid extraction and supercritical fluid extraction.

B. Rodríguez-Morgado · C. Santa-María · E. Revilla · J. Parrado ·
A. Castaño (✉)
Departamento de Bioquímica y Biología Molecular, Universidad de
Sevilla, C/Profesor García González, 2, 41012 Sevilla, Spain
e-mail: angelica@us.es

B. Gordillo
Food Colour & Quality Lab, Department of Nutrition & Food
Science. Facultad de Farmacia, Universidad de Sevilla,
41012 Sevilla, Spain

M. Candiracci
Brigham and Women Hospital, Thorn 13, Anesthesia. 75 Francis St,
Boston, MA 02115, USA

Grape polyphenols possess many biological activities, including anti-inflammatory properties [6] that can be beneficial to human health. It is now widely accepted that inflammation plays a pivotal role in neurodegenerative processes like Parkinson's disease or Alzheimer's disease. The hallmark of neuroinflammation is the activation of microglia, the brain's major defense against immune challenge. However, activated microglia may also contribute to neurodegeneration through the release of pro-inflammatory and/or cytotoxic factors such as IL-1 β , TNF- α , nitric oxide (NO) and reactive oxygen intermediates, among others. It can therefore be assumed that a certain degree of brain inflammation is required to repair the damaged tissue yet excessive inflammation causes neuronal cell death. It would be interesting, therefore, to search for molecules that could help control inflammation in the CNS [7–10]. Natural polyphenols have been shown to exert neuro-protective properties by inhibiting the release of pro-inflammatory cytokines after LPS-activation of microglia [11–14].

This paper describes a new extraction method based on enzymatic technology for obtaining a valuable product from GP. The chemical and functional characterization of the enzymatic extract GP-EE, focusing on its anti-inflammatory properties, is also described.

Materials and Methods

Enzymatic Hydrolysis

GP was resuspended in water at a 20 % concentration. Enzymatic hydrolysis was performed using an endoprotease mixture (0,3 % *v/v*) in a bioreactor using the pH-stat method at 60 °C and pH 8. The solids were then removed by filtration and the final product (GP-EE) was concentrated using a rotary evaporator, obtaining a completely water-soluble syrup. The concentrated extract was lyophilized to obtain a fine red powder with a phenolic yield of 12 % of dry matter.

GP-EE Chemical Composition

The chemical composition of GP-EE, as protein, fat, carbohydrates and fiber, was characterized by using AOAC standard protocols [15]. Molecular-mass protein distribution in GP-EE was determined by size-exclusion chromatography using an ÄKTA-purifier (GE Healthcare) according to the procedure described by Bautista [16], using a Superdex PeptideTM 10/300 GL column (optimum separation range 0.1–7 kDa). Total phenolics were determined using a modification of the Folin-Ciocalteu method [17].

Phenolic Composition by UPLC

The analysis of the individual phenolics was undertaken *via* ultra-high performance liquid chromatography (UPLC) using Agilent 1260 system equipment with a diode-array detector. Phenols were separated on a C18 Poroshell 120 column (2.7 μ m particle size, 5 cm \times 4.6 mm) (Agilent, Palo Alto, CA) and maintained at 25 °C. The solvents used were water-formic acid (99:1, *v/v*) as solvent A, and acetonitrile as solvent B. The flow rate was 1.5 mL/min and the injection volume was 30 μ L. The linear gradient elution was: 0 min, 100 % A; 5 min, 95 % A and 5 % B; 20 min, 50 % A and 50 % B; 22 min, 100 % A; 25 min, 100 % A. The detection wavelengths were 280 nm (flavanols and benzoic acids); 320 nm (cinnamic acids and their derivatives) and 370 nm (flavonols). The analyses were performed in triplicate for each sample.

Individual phenols were identified by comparing their retention time and spectra against standards. The external calibration method was used for quantification comparing the areas with standards of gallic, protocatechuic, caffeic acid, catechin, epicatechin, quercetin-3-O- glucoside and kaempferol-3-O-glucoside. All standards were HPLC-grade and purchased from Sigma-Aldrich (Madrid, Spain).

Phenolic compounds which were not available as standards were identified by their retention time and spectra, according to the literature. These compounds have been assayed by assuming that their molar absorptivity is the same as that of the corresponding free standard molecule.

N13 Cell Culture and Immunostimulation Assays

After stimulation with LPS, N13 microglia produces a repertoire of cytokines similar to primary microglia [18]. Cells were grown in RPMI 1640 (PAA, Linz, Austria) supplemented with 2 mM glutamine (PAA), 5 % (*v/v*) fetal bovine serum (PAA), 100 U/mL 131 penicillin, and 100 μ g/mL streptomycin (PAA) at 37 °C and 5 % CO₂. For subculture, cells were removed from the culture flask with a scraper, resuspended in the culture medium, and subcultured in 6-well plates for experiments (Nunc, Thermo Fisher Scientific, USA) in culture medium at a density of 5.0×10^5 cells/well/2 mL. After adhering, cells were stimulated with LPS (0.01 μ g/mL) and simultaneously different concentrations of GP-EE were added (0, 1, 5 and 10 μ g/mL of polyphenols). Finally, they were collected at different times after stimulation (1, 4 and 6 h), to extract RNA and proteins. Cells treated only with cell culture media but no GP-EE or LPS were used as control.

Determination of Cell Viability

After 24 h of GP-EE treatment (0, 1, 5 and 10 μ g/mL of polyphenols), cells were washed with 1 mL of PBS and stained for 60 min with 1 mL of a 1 % crystal violet solution.

After careful aspiration of the crystal violet solution, the plates were washed with deionized water and dried prior to the solubilization of the bound dye with 1 mL of a 1 % aqueous SDS solution. The optical density of the plates was measured at 590 nm in a microplate spectrophotometer.

RNA Extraction and Reverse Transcription

For PCR analysis, total RNA was extracted from collected cells using the TripureTM Isolation Reagent (Roche, Germany), following the manufacturer's instructions. This procedure allows the isolation of total RNA, DNA and protein fractions from a single sample. Reverse transcription (RT) was performed using random hexamers primers, 3 µg of total RNA as a template and the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer's recommendations as previously described [19].

Real-Time PCR

After RT, the cDNA was diluted in sterile water and used as template for amplification by the polymerase chain reaction. For real time RT-PCR, each specific gene product was amplified using commercial TaqManTM probes employing the ABI Prism 7000 sequence detector (Applied Biosystems, Madrid, Spain) as previously described [18]. The cDNA levels were determined using GAPDH as housekeeper. Threshold cycle (Ct) values were calculated using the software supplied by Applied Biosystems.

Immunoblot

Protein pellets obtained using the TripureTM isolation reagent were resuspended in 4 % SDS and 8 M urea in 40 mM Tris-HCl. The total recovery and integrity of these fractions were determined by Lowry *et al.* [20] and SDS-polyacrylamide gel electrophoresis. Immunoblots were performed [21]. Membranes were incubated overnight at 4 °C with the rabbit polyclonal antibody antiinOS (BD Bioscience, USA, 1/1000) or the mouse monoclonal antibody against β-actin (Sigma-Aldrich) at a dilution of 1/10,000. The blots were developed using the ECL-plus detection method (Amersham).

For quantification, the optical density of the individual bands was analysed using PCBAS 2.08 software (Raytest Inc, Berlin, Germany) and the optical density of each band was normalized relative to the optical density of β-actin.

Statistics

Data were expressed individually, as mean±SD or as a percentage with respect to control.

At least three independent experiments were conducted and analyzed statistically using *t*-student Bonferroni or analysis. Different levels of significance (**p*<0.05 and ***p*<0.001) are considered statistically significant.

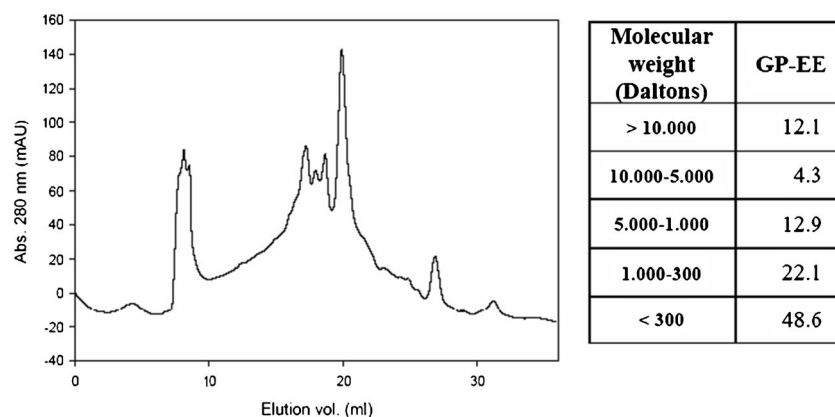
Results and Discussion

Obtaining the Grape Pomace Enzymatic Extract (GP-EE)

GP is a water-insoluble solid by-product composed mainly of fiber (54 %) and minor components such as fat (7 %), proteins (9 %) and soluble carbohydrates (14 %). We have developed a new process using proteases in order to extract and solubilize valuable compounds such as peptides, polyphenols, *etc.* In this process, the strategy consisted of breaking down cell structure, thus solubilizing the protein. This led to the release of molecules such as phenolics or peptides. Enzymatic extraction processes using carbohydrate hydrolase (cellulase and pectinase) enzymes have been described previously [2, 22], but not the use of proteases. Therefore not only have we tried to recover phenolics but also other functional biomolecules with anti-inflammatory and antioxidant capacities such as peptides. The other enzymatic processes hydrolyzed structural carbohydrates, focusing mainly on the recovery of phenols. Due to the fact that the stability of the active ingredients is enhanced at low temperatures and pressures, the biological extraction process employing hydrolytic enzymes has certain advantages over classical chemical extraction processes. The enzymatic extraction method excludes the use of xenobiotics or toxic reagents, something that must also be taken into account, as it is more environmentally friendly.

Due to their fiber compounds (cellulose, hemicellulose, pectin and lignine) carbohydrates, which are the main component in GP-EE (47 %), are not extracted by the enzymatic treatment and remain insoluble and are separated from GP-EE by centrifugation. The second main compound in GP-EE is protein (14 %) which is present in a greater amount than in GP. This is due to the use of proteases which extract, solubilize and hydrolyse the initial insoluble proteins. The physical properties of the proteins in GP-EE are somewhat different to those of GP proteins, as shown in Fig. 1. In GP-EE, the proteins are mainly in the form of peptides and free amino acids. This change in the protein size modifies drastically water solubility, thus the components of GP-EE are completely soluble, independent of the pH. This results in an increase in nutritional functionality. Phenols (12 %), that have been concentrated due to the enzymatic process, are the third component. Although the amount of polyphenols in the GP-EE is similar to that found by other studies [2], our target was not only to obtain a phenolic extract but also one that contains polyphenols and other bioactive molecules such as peptides, carbohydrates and lipids in a soluble form.

Fig. 1 Size-exclusion chromatography and molecular weight distribution of GP-EE



Finally, the fat content is greatly reduced but it is solubilized due to protein interactions. A similar process of lipid extraction and solubilization (emulsion) by proteases from a natural organic matter has also been described [23].

The next goal was to analyze the presence of bioactive compounds that may be involved in the beneficial activities of GP-EE. Using bibliographical data, we have devised a series of bioactive compounds with anti-inflammatory, antitumoral and antioxidant properties that are present in grape seeds and skin, such as catechin, epicatechin, gallic acid, kaempferol, procyanidin B1, procyanidin B2, quercetin and resveratrol. Although a similar grape pomace enzymatic extract has been already characterized [24], we have performed an in-depth characterization of the polyphenolic and protein profile of our GP-EE. Interestingly, present results suggest that the enzymatic extract of grape pomace (GP-EE) comprises a rich source of bioactive non-colored polyphenols (12 %), comprising mainly flavonoids (flavanols and flavonols) and phenolic acids.

Flavonoids are important for human health because of their high levels of pharmacological activity as radical scavengers

[25]. Three flavanols, six flavonols and four phenolic acids were identified and quantified by UPLC (Table 1). Among the flavanols detected, epicatechin and catechin were the most abundant compounds (35.7 and 33.7 mg/100 g, respectively), while procyanidin B1 was also present in high amounts (21.6 mg/100 g). The main flavonols were quercetin 3-*O*-glucoside, kaempferol 3-*O*-glucoside and quercetin 3-*O*-rutinoside (50.9, 21.9 and 20.5 mg/100 g, respectively). Although in lower amounts, three *O*-glycosides from the aglycones quercetin and kaempferol were also found in the enzymatic extract.

With respect to the phenolic acids, both hydroxybenzoic (gallic and protocatechuic) and hydroxycinnamic (caffeic and caftaric) acids were detected in extracts. Protocatechuic acid was the most abundant phenolic acid (27.1 mg/100 g), although the other phenolic acids were also detected in considerable quantities.

Therefore, polyphenols, including both hydrophobic and hydrophilic groups that interact with proteins and polysaccharides on the cell wall, can be released after treatment with proteases.

Table 1 Detailed phenolic composition and total phenolic content of GP-EE (mg/100 g DW)

Compound	Phenolic family	λ (nm)	Concentration (mg/100 g)
Gallic acid	Phenolic acid	280	9.43±0.42
Caffeic acid	Phenolic acid	320	9.69±0.10
Caftaric acid	Phenolic acid	320	16.32±0.13
Protocatechuic acid	Phenolic acid	280	27.07±0.71
Catechin	Flavanol	280	33.74±0.81
Epicatechin	Flavanol	280	35.66±0.59
Procyanidin B1	Flavanol	280	21.62±0.43
Quercetin-3- <i>O</i> -rutinoside (rutin)	Flavonol	370	20.53±0.43
Quercetin-3- <i>O</i> -glucoside	Flavonol	370	50.92±0.04
Kaempferol-3- <i>O</i> -glucoside	Flavonol	370	21.92±0.12
Quercetin 3- <i>O</i> -galactoside	Flavonol	370	6.42±0.31
Kaempferol 3- <i>O</i> -galactoside	Flavonol	370	10.05±0.31
Kaempferol 3- <i>O</i> -glucuronide	Flavonol	370	5.82±0.18
Total phenols (mg/g)		765	12800.13±870

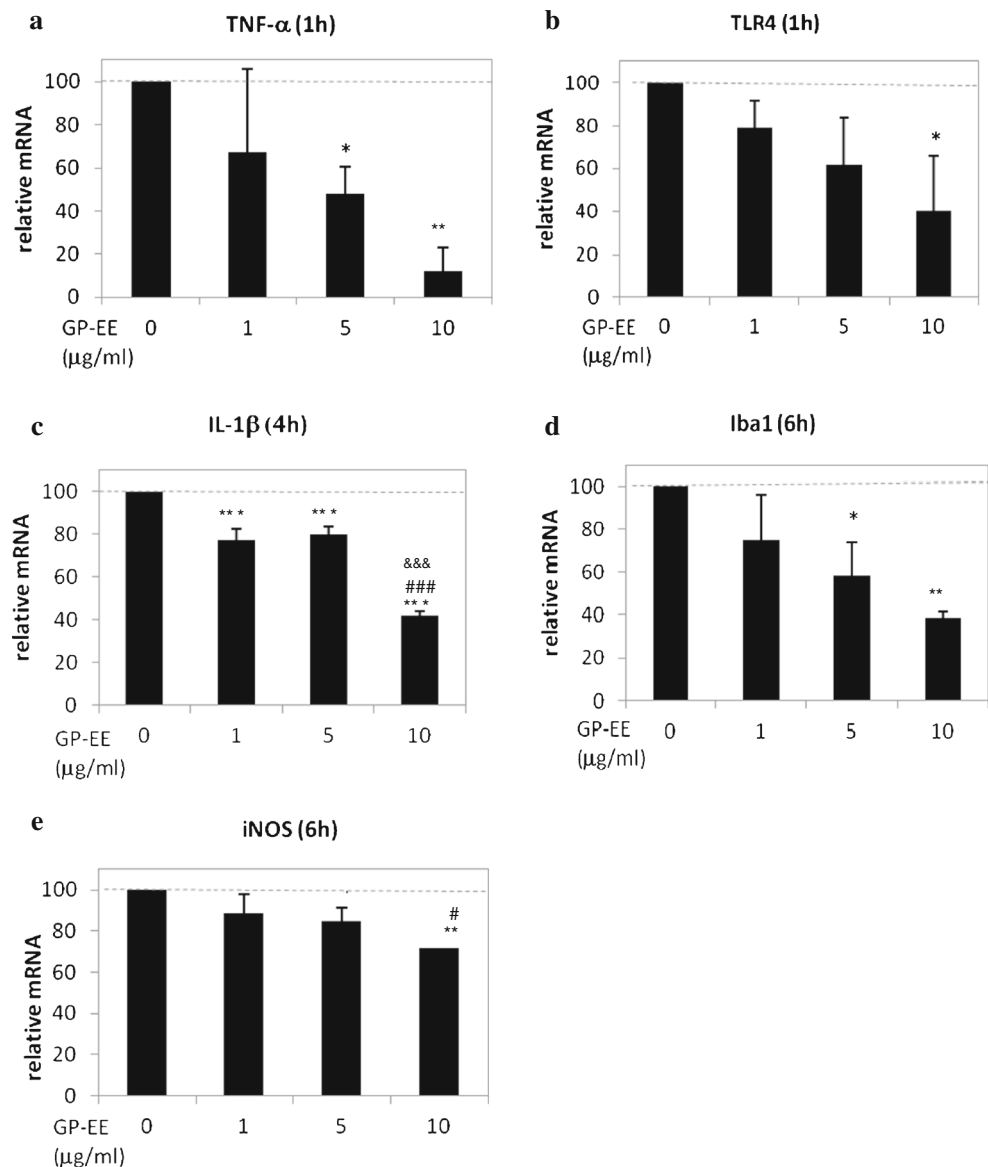
Anti-inflammatory Activity of GP-EE on LPS-activated N13 Cells

The anti-inflammatory property was analysed by *in vitro* assays on N13 cells stimulated with LPS and then treated with different concentrations of GP-EE. It is known that N13 microglia produces a repertoire of cytokines similar to primary microglia [18] after stimulation with the classical pro-inflammatory agent LPS. Accordingly, present results show up-regulation of mRNA expression of pro-inflammatory molecules in N13 microglia at the time points studied (1, 4 and 6 h) after LPS-stimulation. Molecules tested were the cytokines TNF- α , IL-1 β ; the inflammatory enzyme iNOS; the receptor TLR-4 as well as the molecule iba-1. Specifically, mRNA values reached a maximum at hour 1 for TNF- α and TLR-4; hour 4 for IL-1 β , and at hour 6 for iNOS and Iba-1.

To assay whether GP-EE exerts any protective effect on LPS-stimulated cells, N13 cells were simultaneously treated with LPS and different concentrations of GP-EE (0, 1, 5 and 10 $\mu\text{g}/\text{mL}$ on polyphenols). No cytotoxicity was observed for GP-EE treatments at the tested concentrations (data not shown), as tested by cell viability assay. Similarly, GP-EE did not up-regulate the mRNA expression of pro-inflammatory factors when it was added to control cells not stimulated with LPS (data not shown).

Interestingly, GP-EE decreased the up-regulation of the mRNA of all the pro-inflammatory factors studied (TNF- α , TLR-4, IL-1 β , iba-1 and iNOS,) after LPS-stimulation in a dose-dependent manner (Fig. 2). In the presence of GP-EE the expression of TNF- α mRNA was strongly inhibited, showing a significant decrease (50 % with respect to LPS-treated cells) with a medium concentration of GP-EE (5 $\mu\text{g}/\text{mL}$ on

Fig. 2 The protective effect of GP-EE on the expression of mRNA of: TNF- α (a), TLR-4 (b), IL-1 β (c), Iba-1 (d) and iNOS (e) in N13 microglia cells. N13 cells activated with LPS (1 $\mu\text{g}/\text{mL}$) were treated with different concentrations of GP-EE. All data are presented as mean \pm S.D of three independent experiments. Bonferroni analysis was employed to compared the differences between the experimental groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control with LPS alone; # $p < 0.05$ compared with 1 $\mu\text{g}/\text{mL}$ GP-EE and &&& $p < 0.001$ compared with 5 $\mu\text{g}/\text{mL}$ GP-EE



polyphenols) and an even higher decrease (90 % with respect to LPS-stimulated cells; $p < 0.05$; Fig. 2a) using the highest concentration of GP-EE tested (10 $\mu\text{g}/\text{mL}$ on polyphenols).

TLR-4 is the central component of the LPS receptor [26]. Our results show that TLR-4 mRNA also increased after LPS-stimulation. Simultaneous treatment with the highest concentration of GP-EE (10 $\mu\text{g}/\text{mL}$) significantly decreased TLR-4 mRNA to a value of 40.63 % with respect to LPS-treated cells ($p < 0.05$; Fig. 2b), thus also interfering in the expression of the LPS-receptor.

The expression of IL- β mRNA was also significantly inhibited in presence of GP-EE and the decrease was also dose-dependent. There was a decrease of up to 42 % with respect to LPS cells stimulated with the highest concentration of GP-EE (10 $\mu\text{g}/\text{mL}$ on polyphenols; $p < 0.001$; Fig. 2c).

Iba-1 is a microglia/macrophage-specific calcium-binding protein that participates in membrane ruffling and phagocytosis in activated microglia [27]. Accordingly, the activation of Iba-1 mRNA expression was observed after LPS-stimulation (Fig. 2d). Iba-1 mRNA values also decreased with GP-EE with respect to LPS-treated cells, being statistically significant at concentrations corresponding to 5 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$ of polyphenols (75 and 38 % with respect to LPS-stimulated cells; $p < 0.05$).

Finally, we tested the pro-inflammatory enzyme iNOS. GP-EE also decreases the upregulation of iNOS mRNA after the LPS-stimulation of N13, but the effect was less pronounced than that described above, reaching values of up to 84.6 % ($p < 0.05$) and 72 % ($p < 0.001$) with regard to that of LPS-stimulated culture (Fig. 2e) at GP-EE concentrations of 5 and 10 $\mu\text{g}/\text{mL}$ of polyphenols respectively. Interestingly, analysis of protein isolated from LPS-stimulated N13 cells also confirmed a decrease in iNOS protein expressions on LPS-stimulation when compared with cells treated with GP-EE. Our results (Fig. 3) show an approximately 40 % inhibition of iNOS protein by GP-EE at doses of 5 and 10 $\mu\text{g}/\text{mL}$ polyphenols ($p < 0.05$).

In conclusion, GP-EE exerts a clear anti-inflammatory effect *in vitro*, decreasing all of the pro-inflammatory factors

that characterize microglial activation. These results coincide or agree with those of other research groups. Indeed, a similar finding has recently been reported and thus nature molecules such as flavonoids and tocopherols inhibit the release of pro-inflammatory cytokines after activating microglia with LPS [11–13].

Catechin is one of the most abundant flavanols in GP-EE. It has been reported that catechin isolated from *Juniperus rigida* effectively inhibits LPS-induced NO production in RAW264.7 macrophage cells [28]. We can, therefore, suggest that the anti-inflammatory properties of GP-EE make it an ideal source of ingredients to be used as a preventive for neurodegenerative processes. In this regard, wine consumption has been reported as potentially protecting against certain neurological disorders and that its neuroprotective activity is related to its polyphenolic content [29]. However, we must not overlook the fact that other components of GP-EE may also contribute to its anti-inflammatory properties. In fact, the second main component in GP-EE is protein (14 %), mainly in the form of peptides and free amino acids and it is known that vegetable peptides, such as the lunasin from soybean, exert anti-oxidant and anti-inflammatory properties [30].

Neuroinflammation is an important contributor to the pathogenesis of neurodegenerative disorders such as Alzheimer's or Parkinson's disease, both of which present an increasing health problem in modern society [7–10]. One of the hallmarks of neurodegeneration is the presence of activated microglia, the resident macrophages in the central nervous system, and the first line of defense after tissue damage and/or infection. However, activated microglia may also contribute to neurodegeneration through the release of pro-inflammatory and/or cytotoxic factors such as IL- 1β , TNF- α , NO and reactive oxygen intermediates among others. Therefore, after an initial insult microglia activation damages neurons that then release toxic soluble factors. These in turn induce microglial activation, termed as reactive microgliosis. Thus, a self-perpetuating cycle causing prolonged inflammation is created [7, 8]. In this context it has been proposed that regulating excessive microglial activation may be therapeutic in

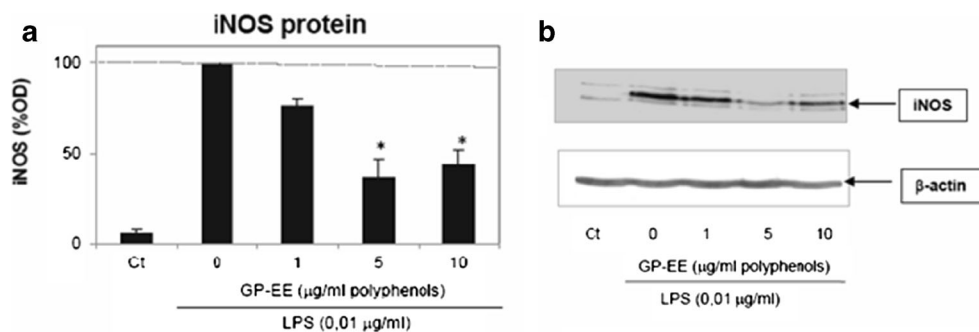


Fig. 3 Inhibitory effect of GP-EE on the expression of the iNOS protein in LPS-stimulated N13 cells. Bands were scanned and the relative density of iNOS (iNOS/ β -actin) was calculated (a). Results are mean \pm SD of

three different experiments. T-student analysis was employed ($p < 0.05$ vs control). The lower panel shows a representative immunoblot in which iNOS is detected (b)

neurodegenerative diseases [31]. Therefore, we can suggest that the anti-inflammatory properties of GP-EE make it an ideal source of ingredients to be used as chemopreventive agents in neurodegenerative disorders linked to neuroinflammation such as Alzheimer's or Parkinson's disease — a growing health concern in modern societies [7–10].

The potential use of polyphenols for the prevention/treatment of neurological disorders is largely hindered by their complexity and also by the limited knowledge regarding their bioavailability, metabolism, and bioactivity, especially in the brain. However, Wang *et al.* [32] have recently demonstrated that after dietary supplementation with polyphenolic preparations from grape, bioactive polyphenol metabolites selectively reach and accumulate in the brain. In a mouse model of Alzheimer's disease, these polyphenolic preparations significantly mitigate amyloid-(A β) mediated neuropathology and cognitive impairments.

In conclusion, we have developed a biological extraction process that allows grape pomace to be extracted with several advantages such as high stability and high content of bioactive compounds such as polyphenols and peptides, while avoiding the use of xenobiotics or toxic reagents. The results strongly support that this extract possesses anti-inflammatory properties that have been widely linked to polyphenols. As a result, GP-EE may be of therapeutic interest in neurodegenerative diseases linked to neuroinflammation by acting as a chemopreventive agent. Thus, we propose the use of GP-EE as a natural extract whose implementation in the functional food field could provide health benefits.

Acknowledgments This research was supported by a grant of the Ministerio de Innovación y Ciencia, Spain (TRACE 2009-0263-01).

Conflict of Interest The authors declare no conflicting interests or financial disclosures.

References

1. Rubilar M, Pinelo M, Shene C, Sineiro J, Núñez MJ (2007) Separation and HPLC-MS identification of phenolic antioxidants from agricultural residues: almond hulls and grape pomace. *J Agric Food Chem* 55:10101–10109
2. Ariel L, Fontana R, Antonioli A, Bottini R (2013) Grape pomace as a sustainable source of bioactive compounds: extraction, characterization, and biotechnological applications of phenolics. *J Agric Food Chem* 61:8987–9003
3. Luque-Rodríguez JM, Pérez-Juan P, Luque de Castro MD (2006) Extraction of polyphenols from vine shoots of *Vitis vinifera* by superheated ethanol-water mixtures. *J Agric Food Chem* 54:8775–8781
4. Monagas M, Hernández-Ledesma B, Gómez-Cordovés C, Bartolomé B (2006) Commercial dietary ingredients from *Vitis vinifera* L. leaves and grape skins: antioxidant and chemical characterization. *J Agric Food Chem* 54:319–327
5. Kashif Ghafoor K, AL-Juhaimi FY, Choi YE (2012) Supercritical fluid extraction of phenolic compounds and antioxidants from grape (*Vitis labrusca* B.) seeds. *Plant Foods Hum Nutr* 67:407–414
6. Xia EQ, Deng GF, Guo YJ, Li HB (2010) Biological activities of polyphenols from grapes. *Int J Mol Sci* 11:622–646
7. McGeer PL, McGeer EG (2004) Inflammation and the degenerative diseases of aging. *Ann N Y Acad Sci* 1035:104–116
8. Gao HM, Hong JS (2008) Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. *Trends Immunol* 29:357–365
9. Block ML, Zecca L, Hong JS (2007) Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* 8: 57–69
10. Lull ME, Block ML (2010) Microglial activation and chronic neurodegeneration. *Neurotherapeutics* 7:354–365
11. Candiracci M, Piatti E, Dominguez-Barragán M, García-Antrás D, Morgado B, Ruano D, Gutiérrez JF, Parrado J, Castaño A (2012) Anti-inflammatory activity of a honey flavonoid extract on lipopolysaccharide-activated N13 microglial cells. *J Agric Food Chem* 60(50):12304–12311
12. Bi XL, Yang J, Dong YX, Wang JM, Cui YH, Ikeshima T, Zhao YQ, Wu CF (2005) Resveratrol inhibits nitric oxide and TNF-alpha production by lipopolysaccharide-activated microglia. *Int Immunopharmacol* 5:185–193
13. Lu X, Ma L, Ruan L, Kong Y, Mou H, Zhang Z, Wang Z, Wang JM, Le Y (2010) Resveratrol differentially modulates inflammatory responses of microglia and astrocytes. *J Neuroinflammation* 7:46–60
14. Ha SK, Moon E, Kim S (2010) Chrysin suppresses LPS-stimulated proinflammatory responses by blocking NF- κ B and JNK activations in microglia cells. *Neurosci Lett* 485:143–147
15. AOAC (1990) Official methods of analysis, 14th edn. DC. Association of Official Analytical Chemists, Washington
16. Bautista J, Hernandez-Pinzón I, Alaiz M, Parrado J, Millan F (1996) Low molecular weight sunflower protein hydrolysate with low concentration in aromatic acids. *J Agric Food Chem* 44:967–971
17. Singleton VL, Rossi JA Jr (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16:144–158
18. Righi M, Mori L, De Libero G, Sironi M, Biondi A, Mantovani A, Donini SD, Ricciardi-Castagnoli P (1989) Monokine production by microglial cell clones. *Eur J Immunol* 19:1443–1448
19. Gavilán MP, Castaño A, Torres M, Revilla E, Caballero C, Jiménez S, García-Martínez A, Parrado J, Vitorica J, Ruano D (2009) Age-related increase in the immunoproteasome content in rat hippocampus: molecular and functional aspects. *J Neurochem* 108:260–270
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265–275
21. Ruano D, Revilla E, Gavilán MP, Vizuete ML, Pintado C, Vitorica J, Castaño A (2006) Role of p38 and iNOS in the in vivo dopaminergic cells degeneration induced by inflammatory processes after LPS injection. *Neuroscience* 140:1157–1168
22. Kammerer D, Aus A, Schieber A, Carle R (2005) A novel process for the recovery of polyphenols from grape (*Vitis vinifera* L) pomace. *J Food Sci* 70:157–163
23. Gbogouri GA, Linder M, Fanni J, Parmentier M (2006) Analysis of lipids extracted from salmon (*Salmo salar*) heads by commercial proteolytic enzymes. *Eur J Lipid Sci Technol* 108:766–775
24. Rodríguez-Rodríguez R, Justo ML, Claro CM, Vila E, Parrado J, Herrera MD, Alvarez de Sotomayor M (2012) Endothelium-dependent vasodilator and antioxidant properties of a novel enzymatic extract of grape pomace from wine industrial waste. *Food Chem* 135:1044–1051

25. Yao H, Jiang YM, Shi J, Tomás-Barberán FA, Datta N, Singanusong R, Chen SS (2004) Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* 59:113–122
26. Beutler B (2002) TLR-4 as the mammalian endotoxin sensor. *Curr Top Microbiol Immunol* 270:109–120
27. Ito D, Tanaka K, Suzuki S, Dembo T, Fukuuchi Y (2001) Enhanced expression of Iba-1, ionized calcium-binding adapter molecule-1, after transient focal cerebral ischemia in rat brain. *Stroke* 32:1208–1215
28. Jeong EJ, Seo H, Yang H, Kim J, Sung SH, Kim YC (2012) Anti-inflammatory phenolics isolated from *Juniperus rigida* leaves and twigs in lipopolysaccharide-stimulated RAW264.7 macrophage cells. *J Enzym Inhib Med Chem* 27:875–879
29. Gómez-Serranillos MP, Martín S, Ortega T, Palomino OM, Prodanov M, Vacas V, Hernández T, Estrella I, Carretero ME (2009) Study of red wine neuroprotection on astrocytes. *Plant Foods Hum Nutr* 64: 238–243
30. Hernández-Ledesma B, Hsieh CC, de Lumen BO (2013) Chemopreventive properties of Peptide Lunasin: a review. *Protein Pept Lett* 20(4):424–432
31. Ransohoff RM, Perry VH (2009) Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol* 27:119–145
32. Wang J, Weina B, Chen A, Freire D, Vempati P, Zhao W, Gong B, Janle EM, Chen T-Y, Ferruzzi MG, Schmeidler J, Ho L, Pasinetti GMJ (2014) Targeting multiple pathogenic mechanisms with polyphenols for the treatment of Alzheimer's disease experimental approach and therapeutic implications. *Front Aging Neurosci* 6:42. doi: [10.3389/fnagi.2014.00042](https://doi.org/10.3389/fnagi.2014.00042)