Winemaking by-products as anti-inflammatory food ingredients

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ABSTRACT
The anti-inflammatory potential of grape stem (GTE) and grape seed extracts (GSE) obtained by pressurized liquid extraction (PLE) was tested in vitro using THP-1 human macrophage cells (THP-1/M). THP-1/M were activated by lipopolysaccharide (LPS) or human ox-LDL and the cytokine secretion (TNF-α, IL-1β, IL-6 and IL-10) was evaluated. Results indicated that both GTE and GSE, at 10 µg/mL, markedly suppressed the production of pro-inflammatory cytokines when THP-1/M were activated with human ox-LDL. However, when LPS was used to activate THP-1/M, only GTE was able to decrease IL-6 production. HPLC analysis of extracts indicated that GSE presented a high amount of proanthocyanidins, which have been described as anti-inflammatory agents. Meanwhile, GTE contained an important quantity of trans-resveratrol and quercetin derivatives, components which also presented a high anti-inflammatory activity. These results increased the added value of winemaking by products as a source of natural anti-atherogenic ingredients.

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Grape stem; grape seeds; PLE; anti-inflammatory activity; cytokines

1. Introduction
The inflammation process is a complex response of the immune system induced by a variety of external factors (microbial infection) or tissue injury: ischemic, toxic or autoimmune. Early stages of this process include the activation of macrophages that secrete several mediators such as cytokines with pro-inflammatory effect, as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and anti-inflammatory, as IL-10 (Barton, 2008; Zhang, 2008). An acute inflammatory response recovers physiological homeostasis in a short period, while in an extended inflammatory response, due to pathological diseases, it triggers a chronic inflammatory response that usually causes further damage. Usually inflammatory processes induce cardiovascular diseases such as atherosclerosis, diabetes, cancer, rheumatoid arthritis or neurodegenerative diseases (Medzhitov, 2008).

Atherosclerosis consists of cholesterol deposition in the intima of large and medium size arteries, accompanied by a chronic inflammatory process (Barter, 2005). Several studies support the key role of oxidized low-density lipoproteins (ox-LDL) in the early inflammatory and more advanced stages of the atherosclerosis lesions. Ox-LDLs are not
recognized by the LDL receptor apo (B/E), but taken up in a non-regulated manner by the scavenger receptors in monocytes-macrophages and endothelial cells. This process leads to the accumulation of cholesterol in the macrophages, forming foam cells, the hallmark of the atherosclerosis lesion (Chouinard, Grenier, Khalil, & Vermette, 2008). In addition, these ox-LDLs can induce the expression of adhesion molecules, pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) and other mediators of inflammation in macrophages and endothelial cells (Kaperonis, Liapis, Kakisis, Dimitroulis, & Papavassiliou, 2006).

Over the last few years, the recovery of biologically active compounds from agro-industrial by-products is seeing increasing attention, since these wastes traditionally were discharged or employed as animal food or fertilizer (Laufenberg, Kunz, & Nystroem, 2003). In this context, the utilization of winemaking by-products for the production of high added value natural products has emerged, due to the presence of certain phenolic compounds in grapevines (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012). Winemaking by-products have been extensively investigated as sources of antioxidant phenolic compounds (Louli, Ragousis, & Magoulas, 2004; Makris, Boskou, & Andrikopoulos, 2007) with several potential health benefits, such as preventative effects for cardiovascular disease (Leifert & Abeywardena, 2008) and neuro-protective activities for Alzheimer’s disease (Asha, Jolita, & Ishii, 2006). However, little attention was paid to its anti-inflammatory activity. Chu, Tang, Huang, Hao, and Wei (2016) reported that a GSE containing 85% of proanthocyanidins suppressed the mRNA expression of pro-inflammatory cytokines like TNF-α and IL-1β, while mRNA level of IL-10 was greatly promoted in RAW264.7 macrophages. Besides, Silvan, Mingo, and Martinez-Rodriguez (2017) indicated that a grape seed extract modulates campylobacter pro-inflammatory response in human epithelial cell lines. In addition, Tao, Ding, Gao, and Sun (2016) indicated that resveratrol repressed the expression of TNF-α, IL-1β and IL-6 in a cell model of microglia.

Solid–liquid extraction (SLE) is the most traditional technology used to extract the phenolic compound from wine by-products. It is widely known that higher temperatures favor the solubility of the solute in the solvent and thus improve its recovery. Nevertheless, the SLE temperature is limited by the solvent boiling and in some cases due to the loss of volatile compounds. In this regard, pressurized liquid extraction (PLE) allows the use of solvents in a liquid state at higher temperatures. Furthermore a compression effect is made on vegetal particles, which also contributes to improving extraction yield. Moreover, lower amount of solvent is required, extraction is faster, higher yields are attained and loss of volatiles is minimized (Mendiola, Herrero, Cifuentes, & Ibañez, 2007). Thus, several studies proposed PLE extraction as an alternative to conventional solid/liquid extraction in order to obtain phenolic compounds from different winery by-products (Domínguez-Perles, Teixeira, Rosa, & Barros, 2014; Yilmaz & Toledo, 2006).

The aim of this work was to study the in vitro anti-inflammatory capacity of two winemaking by-products’ PLE extracts. THP-1 human macrophages were activated by lipopolysaccharide (LPS), mimicking a general inflammatory response and by human ox-LDL as a model to determine the anti-inflammatory effect of the extracts in an atherosclerotic environment. Furthermore, the chemical composition of both extracts was evaluated to better understand the role of their composition with respect to their anti-inflammatory activity.
2. Material and methods

2.1. Samples and PLE extraction

Stem and seed samples from red grape variety Merlot (*Vitis vinifera* L.) were provided by Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA, Spain). Stems were obtained after the manual de-stemming process and seeds were manually recovered after grapes’ pressing. Both by-products were dried at 40°C for 48 h in an air bath. The dried material was ground and the resulting powder was sieved to a ≤1 mm particle size and stored to −20°C.

PLEs were carried out in an ASE 350 system from Dionex Corporation (Sunnyvale, USA) equipped with a solvent controller unit. Each extraction cell (11 mL of capacity) was filled with 1 g of grounded samples and 1 g of sea sand, and then placed into an oven. The experimental conditions (solvent, temperature and time) employed in this paper were related to a previous work carried out in the research group. In that work, we studied the best experimental conditions in order to obtain extracts with the highest phenolic content and antioxidant activity. Thus, 30% water-ethanol as solvent, 120°C and 10 min were used to obtain GTE; meanwhile 75% ethanol, 20°C and 11 min were selected conditions for GSE. The extracts were recovered in glass vials; ethanol was eliminated by evaporation and lyophilized. The lyophilized samples were stored at −20°C in the dark until analysis.

2.2. HPLC analysis of phenolic composition.

Chromatographic analyses of phenolic compounds were carried out using a C18 ACE RP18-AR (150 mm × 4.6 mm, 3 µm particle size) (Symta, Spain) protected by a guard column ACE 3 C18-AR (7 mm × 13 mm) packed with the same stationary phase. The temperature of the column oven was set at 30°C and volume sample injection was 40 µl. Solvent (A) was 2% aqueous formic acid and solvent (B) was acetonitrile containing 2% formic acid, with a flow rate of 1 mL/min. The elution program used was a linear gradient as follows: 0 min, 0% B; 40 min, 4% B; 46 min, 9% B; 70 min, 14% B; 80 min, 21% B; 100 min, 40% B; 110 min, 70% B; 115 min, 100% B; 125 min, 100% B; 130 min, 0% B; 140 min, 0% B. The HPLC-PAD analyses were carried out in an Agilent HPLC 1260 series controlled by ChemStation software (Agilent, vers. 6.8) (Agilent Technologies Inc., USA). Previous to injection, all the samples were diluted in 1 mL of ultrapure water:methanol (1:1) and then filtered by a 0.45 µm PVDF filter. Quantification was carried out by external calibration curves with analytical standards. Hydroxybenzoic acids and flavan-3ols were quantified at 280 nm, hydroxycinnamic acids and stilbenes at 320 nm, flavonols at 360 nm and anthocyanins at 520 nm.

2.3. Isolation and oxidation of LDLs

LDLs were isolated from human plasma and oxidized as described before (Arranz, Jaime, López de las Hazas, Reglero, & Santoyo, 2015). Oxidation of LDLs was done by incubating LDLs with 5 µM CuSO₄ for 3 h at 37°C. Oxidation degree was measured as the amount of thiobarbituric acid reactive substances (TBARS) produced.
2.4. Cell culture and treatment

Human THP-1 monocytes (American Type Culture Collection, ATCC) were cultured in RPMI 1640 culture medium (Gibco, Spain) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine and 0.05 mM β-mercaptoethanol at 37°C in 95% humidified air containing 5% CO₂. Cells were collected and plated at a density of 5 × 10⁵ cells/mL in 24-well plates. Differentiation to macrophages (THP-1/M cells) was induced by maintaining the THP-1 cells in the presence of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, Spain) for 48 h. After differentiation, cells were washed with PBS and incubated with 75 µg/mL ox-LDLs or 0.05 µg/mL LPS (Sigma, Spain) in the presence of different concentrations of PLEs for 24 h in an FBS-free medium. Then, the supernatants were frozen at −80°C until analysis. Indomethacin (5 μg/mL), an anti-inflammatory drug, was used as a reference.

2.5. Cytotoxicity assays

The cytotoxic effect of the extracts on THP-1/M cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, Spain), according to a published method (Mosmann, 1983). THP-1/M cells in 24-multiwell plates were incubated with RPMI containing different concentrations of the extracts for 24 h at 37°C. Cells were then washed with PBS and 0.5 mg/ml of MTT were added to each well and incubated 4 h at 37°C. Supernatants were discarded and formazan crystals dissolved in an extraction solution (10% sodium dodecyl sulfate in a mixture of dimethyl formamide and water 1:1 v/v, adjusted to pH 4.7 with acetic acid) overnight at 37°C. Formazan quantification was performed by measuring the optical density at 570 nm using a multiscanner autoreader (Sunrise, Tecan) with the extraction solution as a blank. The data were plotted as dose–response curves, from which the concentration required to reduce 50% the number of viable THP-1/M (CC₅₀) after 24 h of incubation with the different extracts was obtained.

2.6. Quantification of cytokines by ELISA

The release of IL-1β, IL-10, IL-6 and TNF-α was measured in the supernatants of THP-1/M cells treated with ox-LDL or LPS in the presence of different concentrations of extracts using ELISA kits (BD biosciences, Spain), according to manufacturer’s instructions. The color generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise, Tecan).

2.7. Determination of total phenolic content

In order to determine the total phenolic content (TPC), the Folin-Ciocalteau reagent method was applied (Singleton, Orthofer, & Lamuela-Reventos, 1999). 3 mL of ultrapure water was mixed with 50 μl of sample in the proper dilution. Then, 250 μl of Folin-Ciocalteau reagent were added and the mixture was shaken. After 3 min, 750 μL of 20%
Na₂CO₃ solution and 950 µL of ultrapure water were added. The new mixture was shaken and let to react for 2 h, protected from direct sunlight. After this time, the absorbance was measured at 760 nm. The same procedure was employed for the blank (water) and gallic acid standard too. The results were expressed as milligrams of gallic acid equivalents (GAE)/g extract.

2.8. Statistical analysis

All data were expressed as the mean of three determinations ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s and Bonferroni tests, using the Prism program for Windows (Version 5; GraphPad Software, San Diego, CA, USA). P values lower than .05 were considered significant.

3. Results and discussion

3.1. Phenolic characterization of extracts

TPC in PLE extracts showed that GSE (354.02 ± 0.2 mg GAE/g extract) presented almost twice in phenolic compounds content than GTE (185.33 ± 2 mg GAE/g extract). Further, the analysis of the phenolic compounds (monomers and oligomers) of both extracts showed important differences also in extracts’ composition (Table 1). Thus, both extracts presented a similar quantity of phenolic acids. Although GSE contained almost exclusively gallid acid and monogalloil glucoside, GTE presented, besides gallic acid, significant quantities of caftaric, vanillic and syringic acids. In addition, the stilbene trans-resveratrol was only detected in GTE. Regarding to flavan-3-ols, GSE showed a higher amount of these compounds than GTE, mainly epicatechin and catechin. However, the amount of flavon-3-ols in GSE was very low; meanwhile GTE contained mainly quercetin-3-O-glucuronide. These results were consistent with that indicated by other authors, since principal compounds in seed extracts have been reported to be flavanols, mainly as flavan-3-ols, where catechin and epicatechin were the main monomeric compound (Obreque-Slier, López-Solís, Castro-Ulloa, Romero-Díaz, & Peña-Neira, 2012). Montealegre, Peces, Vozmediano, Gascueña, and Romero (2006) also indicated the presence of gallic acid in Merlot seed extracts. Regarding stem extracts, flavan-3-ols and different phenolic acids, mainly gallic and caftaric acids, were also detected (Anastasiadi et al., 2012; Sun & Spranger, 2005). In addition, trans-resveratrol has been previously described as a stilbene present in stem extracts (Püssa, Floren, Kuldkepp, & Raal, 2006).

3.2. Effects of extracts on THP-1/M viability

PLE grape stem (GTE) and grape seed (GSE) extracts were initially evaluated for cytotoxicity on THP-1/M cells by the MTT method. The CC₅₀ data obtained indicated that GTE presented a lower cytotoxicity (84.70 ± 5.32 µg/mL) than GSE (72.63 ± 4.23 µg/mL). However, at the higher concentration used in the anti-inflammatory assays, 20 µg/mL, both extracts presented no cytotoxicity. In addition, indomethacin at 5 µg/mL presented no cytotoxicity.
3.3. Effect of GTE and GSE on the cytokines’ release in THP-1/M activated with ox-LDL or LPS

In a first assay, the activation of THP-1/M was carried out with the addition of ox-LDLs to the medium. These ox-LDL-treated cells showed, after an incubation period of 24 h, a significant increase in the release of all pro- and anti-inflammatory cytokines tested (TNF-α, IL-1β, IL-6 and IL-10) compared to non-activated controls (Figure 1). These activated cells were considered as positive controls for all the cytokines tested. When the activation of THP-1/M was carried out in the presence of different concentrations (5, 10 and 20 µg/mL) of GTE and GSE, a decrease in TNF-α secreted level was observed (Figure 1), compared with levels obtained in the absence of these extracts (positive control). Moreover, a very significant decrease in the amount for TNF-α secreted was obtained with 20 µg/mL of extracts. At this concentration, GTE produced an inhibition in TNF-α secretion similar to that obtained with 5 µg/mL of Indomethacin. When employing 5 µg/mL of GTE, an inhibition of 40% TNF-α secretion was achieved.

Table 1. Phenolic composition (oligomers/monomers) of PLE stem and seeds extracts (mg compound/g dry extract).

| Phenolic Composition | Stem extract | Seeds extract |
|---------------------|--------------|---------------|
| Gallic acid         | 0.541 ± 0.029 | 1.033 ± 0.003 |
| Protocatechuic acid | 0.008 ± 0.000 | 0.007 ± 0.000 |
| Monogalloyl glucoside | Tr         | 0.434 ± 0.006 |
| 4-Hydroxybenzoic acid | 0.048 ± 0.001 | Nd           |
| Vanillic acid       | 0.224 ± 0.010 | Nd           |
| 2,3-dihydroxybenzoic acid | 0.058 ± 0.004 | Nd           |
| Syringic acid       | 0.202 ± 0.015 | Nd           |

Note: Tr: traces; Nd: no detected; Co: coelute.
Meanwhile at the same concentration GSE was not effective. Regarding IL-1β secretion by activated cells in the presence of these extracts, a significant decrease in the secretion of this cytokine can also be observed. Thus, in this case, 5 µg/mL of the extracts

**Figure 1.** Levels of TNF-α, IL-1β, IL-6 and IL-10 secreted by THP-1/M activated with ox-LDL in the presence of different concentrations of stem and seed extracts. Each bar is the mean of three determinations ± standard deviation. * denotes statistical differences between control + the other samples at $p < .05$. 

Meanwhile at the same concentration GSE was not effective. Regarding IL-1β secretion by activated cells in the presence of these extracts, a significant decrease in the secretion of this cytokine can also be observed. Thus, in this case, 5 µg/mL of the extracts
presented a similar decrease in IL-1β release than indomethacin (40% approx.). With this cytokine, GTE and GSE presented a similar activity. The activation of macrophages in the presence of extracts also produced a significant decrease in the IL-6 release; thus 10 and 20 µg/mL of both extracts were able to decrease the IL-6 release to basal levels (negative control). Moreover, 5 µg/mL of GTE also decreased the IL-6 secretion to basal levels; meanwhile 5 µg/mL of GSE only reduced IL-6 release by 47%. With respect to the anti-inflammatory cytokine IL-10, at the concentrations employed, no significant differences were found between positive control and activated cells in the presence of the extracts.

In a second assay, the activation of THP-1/M was carried out with the incorporation of LPS to the cell medium during 24 h, with or without different concentrations of GTE and GSE. As shown in Figure 2, LPS-treated cells increased significantly the secretion of TNF-α, IL-1β, IL-6 and IL-10, compared to non-activated cells. In general, the incorporation of 5, 10 or 20 µg/mL of extracts to activated cells did not modify the release of all cytokines tested, although 20 µg/mL of GTE presented a reduction in IL-6 secretion close to 40%.

When comparing the results obtained in the two models of inflammation, data indicated that extracts presented a greater anti-inflammatory effect in the model including ox-LDL. In this sense, several phenolic compounds, such as quercetin, have been reported to have a protective effect against ox-LDL-induced inflammation in human PBMCs, because this compound inhibited pro-inflammatory cytokine production and down-regulated the activity of inflammatory enzymes (Bhaskar, Shalini, & Helen, 2011). In addition, resveratrol was able to prevent the release of pro-inflammatory mediators by ox-LDL-stimulated macrophages (Vivancos & Moreno, 2008). Besides, resveratrol reduced the serum levels of IL-1β, IL-6 and TNF-α in an atherosclerotic rabbit model (Zordoky, Robertson, & Dyck, 2015). Further, Yamaki, Goto, and Takano-Ishikawa (2007) reported that resveratrol inhibits uptake of ox-LDL into macrophages. This result suggested that resveratrol might act not only by inhibiting oxidation in LDL, but also by the uptake of oxidized LDL by macrophages. Regarding phenolic acids, ellagic acid has been shown to inhibit LDL oxidation (Kong, Mat-Junit, Ismail, Aminudin, & Abdul-Aziz, 2014). However, Chu et al. (2016) also reported that a grape seed proanthocyanidins extract (with more than 85% oligomeric proanthocyanidins) inhibited the LPS-induced inflammatory mediator expression in RAW264.7 macrophages, although concentrations employed in that work were excessively higher than those employed in this study. In the same way, Tao et al. (2016) also reported that resveratrol repressed the expression of IL-1β, IL-6 and TNF-α, and promoted IL-10 in a cell model of microglia stimulated with LPS.

Thus, GTE, with a lower concentration of total phenolic compounds than GSE, presented higher anti-inflammatory activity in THP-1/M stimulated with LDL-ox and was able to inhibit IL-6 secretion in macrophages stimulated with LPS. This activity could be mainly related with the presence of trans-resveratrol, flavan-3-ols (mainly catechin, epicatechin and dimer B1) and several quercetin derivatives in the extract. However, GSE, mostly composed by flavan-3-ols, presented a lower anti-inflammatory activity than GTE. These data could indicate that resveratrol, only detected in GTE, could be related to the higher anti-inflammatory activity presented by this extract, since resveratrol might inhibit both oxidation in LDL and the uptake of oxidized
LDL by macrophages (Yamaki et al. (2007). However, this higher anti-inflammatory activity could also be related to a synergistic effect among the main compounds detected in GTE.

**Figure 2.** Levels of TNF-α, IL-1β, IL-6 and IL-10 secreted by THP-1/M activated with LPS in the presence of stem and seed extracts. Each bar is the mean of three determinations ± standard deviation. * denotes statistical differences between control + the other samples at $p < .05$. 

LDL by macrophages (Yamaki et al. (2007). However, this higher anti-inflammatory activity could also be related to a synergistic effect among the main compounds detected in GTE.
4. Conclusion

Concluding, PLE allowed the production of stem and seed extracts with an important anti-inflammatory activity using an atherosclerotic environment model. These extracts act as effective inhibitors of ox-LDL-induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and therefore have a great potential to be used as natural ingredients in the development of anti-atherogenic products. Besides, these results increased the added value of winemaking by products as a source of natural anti-atherogenic compounds.

Disclosure statement

No potential conflict of interest was reported by the authors.

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