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Green extraction of bioactive compounds from wine lees and their bio-responses on immune modulation using in vitro sheep model

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ABSTRACT

The objective of this study was to apply microwave-assisted extraction using green solvents starting from 3 different wine (white, rosé, and red) lees and to test their bio-response on sheep peripheral blood mononuclear cells proliferation, Bax/Bcl-2 ratio, and cytokines secretion. Wine lees (WL) of local organic farming from white wine, produced with Trebbiano cultivar, rosé and red wine, made with Nero di Troia cultivar, were collected. The WL were subjected to microwave-assisted extraction using 2 green solvents (water and ethanol) in 3 different combinations (water; water/ethanol 1:1 vol/vol; ethanol) with a dry matter-to-solvent ratio of 1:40 (wt/vol) at 4 temperature levels: 50, 100, 150, and 200°C. Sodium carbonate Na_2CO_3 (2 mmol/g of dry weight of lees) was used for increasing the polyphenol extraction yield. A total number of 6 extracts, 2 for each kind of WL investigated, according to their total phenolic content and in vitro antioxidant capacity, were selected to be tested on sheep peripheral blood mononuclear cells, as an animal model. All the WL extracts demonstrated a strong antiproliferative action. On the contrary, the cytokines' profile was mainly dependent on the different winemaking derived WL and the extraction solvent combination procedures. Red WL extract obtained by a combination of water/Na_2CO_3 and tested at 0.8 mg/mL, resulted in an increase of both IL-6 secretion and Bax/Bcl-2 ratio. Data from the present study demonstrated that WL extracts derived from different winemaking and solvent extraction could have a bimodal action on control of inflammatory mediated damage and highlighted the importance for further studies aimed at applying the biorefinery process on by-products to increase their economic value and exploit new derived bioactive compound.

Key words: wine lees, circular economy, green solvent, sustainability

INTRODUCTION

Wine lees (**WL**) represent the least aqueous by-product of the wine industry and consist of a combination of the yeasts, metabolites, and other free phenolic compounds, including free flavonol aglycones and pyranoanthocyanins (Barcia et al., 2014; Dimou et al., 2015). The composition of WL can be influenced by many factors, such as environmental conditions, the land type, grape variety, and the time of aging in the wood barrels. The chemical separation from WL of the phenolic compounds could be considered an integrated winery by-product biorefinery process. Recently, microwave technology has gained increased industrial interest as green technology because it (1) can be applied directly to the desired biomass without any need of solvent or pretreatment (i.e., drying); (2) allows rapid and homogeneous heating; and (3) is adaptable for continuous processes and easily scalable (Filly et al., 2014). The scalability of this technology has been proved in different studies and has shown important advantages over traditional methods (Pardo and Zufía, 2012). Currently, by-products seem to have promising applications being sources of considerable amounts of bioactive components (Schieber et al., 2001), especially polyphenols, such as proanthocyanidins (tannins) or flavonoids (Vasta and Luciano, 2011). The dietary inclusion of natural bioactive compounds positively influences small ruminant productive performance and animal health (Min et al., 2003; Vasta et al., 2019). Moreover, it has been reported that nutraceuticals compounds are able to affect gene expression and signaling processes that can control cell apoptosis, immune modulation, and metabolism (Braicu et al., 2017; Surh, 2003; Barnes, 2008; Agrawal and Mishra, 2010). Exploring the ef-

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fects of phytochemicals extracts on immune cells, ovine neutrophils activated with phorbol myristate acetate showed a reduction of cell adhesion and superoxide dismutase production, indicating an enhancement of their anti-inflammatory properties (Farinacci et al., 2007). Water and hydroethanolic extracts of *Larix decidua*, *Thymus vulgaris*, *Salix alba*, *Sinupret*, *Helianthus annuus*, and *Mangifera indica* can modulate sheep neutrophil immune functions, whereas *Larix decidua*, *Thymus vulgaris*, and *Salix alba* exploit high anti-inflammatory activity (Farinacci et al., 2008). Phytosterols extracted and purified from *Dunaliella tertiolecta* show an antiproliferative and immunomodulatory effect, in terms of cytokines secretion, tested on sheep peripheral blood mononuclear cells (**PBMC**; Caroprese et al., 2012). The anti-proliferative action is confirmed in sheep fed flaxseed during postpartum period, with a subsequent *D. tertiolecta* phytosterols in vitro treatment on PBMC, suggesting their potential application with the purpose of controlling tissue damage caused by excessive inflammatory reactions (Ciliberti et al., 2017b). Furthermore, hydrolyzed lignin, a phenolic compound with an antioxidant effect, results in cytoprotection from apoptosis or necrosis after hydrogen peroxide in vitro exposition, and modulation of cytokine profile decreasing TNF-α and increasing of IL-8 production, which are strictly connected with monocyte activation and antioxidant response pathway (Ciliberti et al., 2020).

No information is available on the effects of WL extracts on sheep immune modulation in vitro. Our hypothesis was that WL extracts obtained with different solvents' combination methods could be introduced as feed ingredients to sustain the sheep immune system during stressors and noninfection stressors exposition. Therefore, the objectives of the present study are (1) extraction of bioactive compounds from 3 different winemaking-derived WL using microwave-assisted extraction with the green solvents, water, and ethanol and their combination at different temperatures, and (2) the evaluation of the effects of bioactive compounds extracted from WL selected, based on their best value of the total phenol content, the ferric antioxidant power or the scavenging activity, on sheep peripheral blood mononuclear cells proliferation, apoptosis proteins, and cytokine secretion after in vitro inflammation challenge.

MATERIALS AND METHODS

Wine Lees Sampling and Preparation

Wine lees from organic farming of local wineries located in Foggia, Italy, were collected from white (**Wh**) wine produced with Trebbiano cultivar, and rosé (**Ro**) and red (**Re**) wine produced with Nero di Troia cultivar. The Wh and Ro WL were obtained through bentonite flocculation, whereas Re WL was achieved by decantation only. Moisture, ash, and volatile solids content of each sample was detected by thermal gravimetric analysis (TGA-701 LECO) according to UNI EN ISO 18134-3 (2015) method.

Conventional Extraction

Lees samples were evaporated at 40°C under vacuum for 20 min to remove residual ethanol, and the resultant material was frozen at −40°C and then freeze-dried. Freeze-dried lees samples were ground with a mortar and pestle and kept in airtight containers at −20°C until analysis. Samples were extracted as described by Mercurio et al. (2007). Briefly, 5 mL of 50% (vol/vol) ethanol was added to 0.5 g of freeze-dried sample and then shaken using a thermostatic orbital shaker (Model OM 11, Ratek Instrument Ltd.) for 60 min at room temperature (**RT**, 25°C). Samples were centrifuged at 3,157 × *g* at 25°C for 10 min, and the supernatant liquid phase was filtered (0.22 μm) and stored at −20°C. Conventional extraction of WL was done in triplicate for each sample.

Microwave-Assisted Extraction of WL

Microwave-assisted extraction (**MAE**) of WL was directly performed on wet samples using a microwave system MARS-6 (CEM Srl) equipped with 12 closed extraction vessels, infrared temperature sensor, and magnetic stirring set at 300 rpm. Pressure and temperature were directly measured in the reference vessel by means a pressure sensor and an optic fiber probe. Two green solvents (water and ethanol) in 3 different combinations (water; water/ethanol 1:1 vol/vol; ethanol) were used for the extraction. According to the moisture content, the volume of WL and solvent was set to maintain a ratio of DM to solvent at 1:40 (wt/vol). The final extraction volume was 40 mL. Moreover, sodium carbonate Na_2CO_3 (2 mmol/g of dry weight of lees) was used for increasing the extraction yield of polyphenols. Four temperature levels were tested: 50, 100, 150, and 200°C. The extraction temperature was maintained for 10 min after a ramp of 5 min. The microwave frequency used for the extraction was 2,450 MHz. At the end of the extraction process, the vessels were cooled down to 40°C using compressed air. Then, the mixture was centrifuged at 3,157 × *g* at 25°C for 10 min and the liquid phase was filtered (0.22 μm). The extracts were then flushed with nitrogen gas and stored in dark at −40°C until further analyses. Extractions were performed in triplicate for each experimental condition.

Total Phenolic Content of WL Extracts

The total phenolic (**TP**) compounds content after MAE was determined using the Folin–Ciocalteu assay, and the results were expressed as mg/g of dry weight of gallic acid equivalents (**GAE**; Singleton and Rossi, 1965). Briefly, Milli-Q water was added to aliquots of the extracts (diluted 1:5, vol/vol), to obtain a final volume of 1.4 mL, and then mixed with 300 μL of freshly prepared Folin–Ciocalteu reagent diluted with water (1:2, vol/vol). The mixture was shaken and allowed to react for 5 min. Then, 300 μL of 20% sodium carbonate in water (wt/vol) was added, and the tubes were shaken. After 60 min of incubation at RT in the dark, the absorbance was measured in a quartz cuvette (1 cm) at 765 nm using a UV–vis spectrophotometer (Cary-60, Agilent). The results were expressed as milligrams of GAE per gram of dried WL (mg of GAE/g of dry weight). All samples were analyzed in triplicate.

In Vitro Antioxidant Capacity Assay of WL Extracts

Measure Ferric Antioxidant Power Assay. The measure of ferric antioxidant power (**FRAP**) reagent was prepared according to Francavilla et al. (2013). Briefly, 20 mL of 2,4,6-Tris(2-pyridyl)-s-triazine (10 mmol/L) in HCl (40 mmol/L) were added to 20 mL of $FeCl_3$ (20 mmmol/L) and 200 mL of acetate buffer (0.3 mmol/L, pH 3.6) and then warmed up to 37°C.

A volume of 100 μL of microwave extract was mixed with 3 mL of FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm after incubation at 37°C for 10 min. The ferric reducing antioxidant power of the samples was determined in triplicate and expressed as micromoles of Trolox equivalents antioxidant capacity (**TEAC**) per gram of the dried WL sample (μmol of TEAC/g of dry weight). A calibration curve was freshly prepared before each assay using a 5-point calibration plot and a Trolox concentration range of 3 to 120 μ*M*.

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) Assay. The scavenging activity of WL extract was detected by the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (**ABTS**) assay according to Francavilla et al. (2013). First, the ABTS stock solution was prepared by a reaction of the colorless ABTS 7 m*M* with 2.45 m*M* of $K_2S_2O_8$. The solution was allowed to stand in the dark for 16 h, meanwhile the blue-green $ABTS^{\cdot+}$ form was generated. Second, the working solution was prepared by adding 1 mL of ABTS stock solution to 88 mL of PBS (5 m*M*, pH 7.4), and then 2 mL of this solution was added to 200 μL of samples. After 6 min, the absorbance was measured at 645 nm and the results were expressed as micromoles of TEAC per gram of samples of dried WL (μmol of TEAC/g of dry weight). A calibration curve (5-point plot) was freshly prepared before each assay from methanol solutions of Trolox ranging from 3 to 1,200 μ*M*.

Qualitative GC-MS of WL Extract. The qualitative analysis of selected extracts was performed by using GC-MS equipment composed of a gas chromatograph GC-7890B (Agilent Technologies) coupled with an ion trap mass spectrometer IT-240 (Agilent Technologies).

The gas chromatograph was equipped with a VF-5ms capillary column (Agilent J&W GC Columns), 30 m × 0.25 mm i.d. × 0.25 μm. The column oven was set at 50°C for 1 min after injection; then the temperature was increased from 50 to 300°C, then with a ramp of 5°C/min and a hold time of 10 min. The purge process was performed at the end of each run, increasing the oven temperature from 300 to 325°C at 10°C/min and a hold time of 1.5 min. The total time for each run was approximately 65 min. The injection volume was 1 μL (split ratio 10:1) with a continuous flow rate of 1 mL/min of ultra-high purity helium (BIP) and an injector temperature of 250°C.

The mass spectrometer electron multiplier voltage was set at 1450 V, and an ionization time of 65,000 μs was used, running in the electron impact mode. The transfer line, ion trap, and manifold temperatures were set at 270, 180, and 70°C, respectively. The mass spectrometer operated in full scan mode (50–700 m/z) with the ionizing voltage at 70 eV. The compounds were identified by matching their mass spectra with National Institute of Standards and Technology mass spectra database.

Animals and Experimental Treatments. Dairy sheep used in this study were located at Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE). In the experimental design, 16 sheep were chosen with a random design, and an in vitro experiment was performed, with 2 different concentration 0.4 mg/mL and 0.8 mg/mL of each WL extract. Before testing, the extracts were dried out in N_2 flow and then resuspended with dimethyl sulfoxide (**DMSO**). All procedures were conducted according to the guidelines of the EU Directive 2010/63/EU (European Union, 2010) on the protection of animals used for experimental and other scientific purposes. The animals were carefully examined by veterinarians throughout the trial to exclude the presence of any signs of disease (protocol number 0002302).

Isolation of PBMC. Blood samples were collected in Na-heparinized vacuum tubes from the jugular vein of sheep, and PBMC were isolated by density-gradient centrifugation according to Wattegedera et al. (2004), modified as previously reported in Ciliberti et al. (2017a). Cells viability was about >96%, measured

by trypan blue dye exclusion using Burker chamber. The proliferative response to WL extracts was determined by bromodeoxyuridine (**BrdU**) incorporation during DNA synthesis. Briefly, 100 μL of PBMC (1×10^6) were plated into 96 U bottoms well plate in quadruplicate. Cells were stimulated by the addition of 50 μL per well of Concanavalin A (**ConA**, 5 μg/mL, final concentration) and LPS (1 μg/mL, final concentration, Sigma-Aldrich), as previously reported in Ciliberti et al. (2020). The PBMC were treated with WL extracts (50 μL/well) at 2 different concentrations (0.4 and 0.8 mg/mL with final concentration of DMSO less than 0.5% for all WL tested). The WL extracts concentrations were chosen according to previous studies of Caroprese et al. (2012) and Ciliberti et al. (2017b, 2019) on PBMC treated with bioactive compounds extracted and purified from *D. tertiolecta* and *Chlorella sorokiniana.* The PBMC challenged with Con A and LPS, and with no addition of DMSO represent positive control. The PBMC unchallenged in presence with only culture medium and with no addition of DMSO represent negative control. The plates were incubated in a humidified incubator with 5% CO_2 at 37°C for 24 h. Cell-free supernatants were collected after spin for 5 min at 1,000 rpm. On cells, BrdU was added (20 μL/mL, 10 μ*M* final concentration), after 18 h of incubation the medium was aspirated and cells were treated with 200 μL of well fixative and denaturing solution for 30 min at RT, following ELISA procedure as reported in the manufacturer's instruction. (Exalpha Biologicals Inc.). The PBMC proliferation was measured using spectrophotometer (Power Wave XS, Biotek), and the reading was set at 450 nm.

Determination of Apoptosis Protein Bax and Bcl-2 in Culture Supernatants. Sheep Bcl-2-associated X protein Elisa kit and a sheep beta cell leukemia and lymphoma 2 ELISA kit (Neoscientific) were used, following the manufacturer's instructions, to determine the pro-apoptotic protein Bax and the anti-apoptotic Bcl-2 concentration in culture supernatants in the presence of WL extracts. The absorbance was read at 450 nm, and data were expressed in nanograms per milliliter.

Determination of Cytokines in Culture Supernatants by ELISA. The determination of IL-6 and IL-1β in PBMC supernatants were determined according to Caroprese et al. (2006) and modified as previously reported in Ciliberti et al. (2017a). The sandwich ELISA was build using specific antibody against bovine IL-6 and IL-1β (Clone 4B6 for IL-6 and Clone 1D4, Serotec Ltd.). All incubations were at 37°C. Cytokines concentrations were obtained using a scalar dilution of recombinant ovine IL-6 (Cusabio Biotech Co.) and recombinant bovine IL-1β (Kingfisher Biotech Inc.). The plates were read at 450 nm by a spectrophotometer (Power Wave XS, Biotek). Data were expressed as ng/mL of IL-6 [interassay CV% = 15; intra-assay CV% = 9; limit of quantification (**LOQ**) = 13.97 ng/mL] or IL-1β (interassay CV% = 8.4; intra-assay CV% = 6.44; LOQ = 3.9 ng/mL).

The concentration of IL-10 and IFN-γ in supernatants of PBMC were determined according to Kwong et al. (2002) and modified as previously reported in Ciliberti et al. (2017a, 2020). The sandwich ELISA was build using a specific antibody against bovine IL-10 and IFN-γ (Clone CC318 for IL-10, Serotec, Clone MCA2112 for IFN-γ, AbD Serotec). The plates were read at 450 nm by a spectrophotometer (Power Wave XS, Biotek). Data were expressed as nanograms per milliliter for IL-10 (interassay CV% = 15; intra-assay CV% = 8; LOQ = 4.88 ng/mL) and picograms per milliliter for IFN-γ (interassay CV% = 15; intra-assay CV% = 8; LOQ = 0.048 pg/mL). All the incubations were at RT.

Statistical Analysis.

All statistical analyses were performed using SAS software (version SAS 9.4; SAS Institute Inc.). Data were expressed as least squares means ± standard error of the mean and tested for normality. The effect of WL extracts on PBMC proliferation, cytokines, and apoptotic protein secretions were tested using one-way ANOVA with analysis of contrasts. Tukey test for multiple comparisons between means was performed. Differences at $P < 0.05$ were considered significant. A multivariate analysis was performed on WL extracts data set to explain differences in TP, TRAP, and ABTS among groups (Wh, Ro, and Re) and to evaluate the main parameter (solvent, temperature, and presence or absence of Na_2CO_3) that played the most important role in determining those differences.

RESULTS

Wine Lees Characteristics

The WL samples were originated from 2 different grape cultivars (Trebbiano and Nero di Troia) and 3 different winemaking procedures (Table 1). The proximate analysis showed that Wh had a higher moisture content (82.62 ± 0.70%, $P < 0.001$) than Ro and Re (65.62 ± 0.05% wet weight and 63.43 ± 0.83% wet weight, respectively). These values were used for defining the solid-to-solvent ratio for the microwave extraction process applied to wet WL. The ash content ranged between 18.00 ± 0.05% of dry weight (in Re) and 11.54 ± 0.10% of dry weight (in Ro), whereas volatile solids, the thermally degradable organic fraction,

Table 1. Chemical composition and total phenols (± SE) content of white, rosé, and red wine lees

Item	Wine lees		
	White	Rosé	Red
Cultivar	Trebbiano	Nero di Troia	Nero di Troia
Winemaking technique	Flottation + Bentonite	Flottation + Bentonite	Decantation
Moisture (% wet wt)	82.62 ± 0.70	65.62 ± 0.05	63.43 ± 0.83
Ash (% dry wt)	17.04 ± 0.41	11.54 ± 0.10	18.00 ± 0.04
Volatile solids (% dry wt)	80.16 ± 4.41	80.12 ± 0.51	77.27 ± 0.20
Total phenols (mg of GAE[1]/g dry wt)	21.92 ± 1.09	38.56 ± 1.93	21.44 ± 0.65

[1]GAE = gallic acid equivalents.

ranged between about 80% of dry weight (in Wh and Ro) and 77.27 ± 0.20% of dry weight (in Re).

The WL samples were also characterized in terms of TP content, extracted with the conventional method starting from freeze-dried samples. The highest TP content was found in Ro WL (38.56 ± 1.93 mg of GAE/g of dry weight, $P < 0.001$), whereas Wh and Re WL showed a similar TP content (21.92 ± 1.09 and 21.44 ± 0.65 mg of GAE/g of dry weight, respectively).

Wine Lees Characterization Using MAE

Microwave extraction was performed on WL samples without any preliminary treatment to figure out a simple and easy method to implement the extraction process. Therefore, a certain amount of wet sample was directly added to the solvent to reach a WL (dry weight) to solvent ratio of 1:40 (wt/vol). The yields of total phenols extracted at different experimental conditions from 3 investigated WL samples are reported in Figure 1. Results showed that high variability is related to solvent, temperature, WL origin, and Na_2CO_3 presence or absence.

Solvent and Temperature Effect

Water/Ethanol (1/1). Water/ethanol 1/1 (vol/vol), without Na_2CO_3, was the best solvent for phenols extraction (Figure 1a). The highest TP yield was achieved from Ro WL extracted with MAE at 200°C for 20 min (85.48 ± 10.26 mg of GAE/g of dry weight). This yield was more than twice the TP value achieved at 50°C with MAE (37.28 ± 4.47 mg of GAE/g of dry weight) and with the conventional method at RT for 60 min (38.56 ± 4.63 mg of GAE/g of dry weight). In general, the increase of temperature was correlated with an increase in TP yield ($r = 0.51$, $P < 0.001$). Higher TP concentration from Ro WL was found at higher extraction temperature. Similar behavior was observed for TP from Wh WL. TP extraction yield was 54.8 ± 3.75 mg of GAE/g of dry weight at 200°C, whereas at 50°C was 21.92 ± 1.47 mg of GAE/g of dry weight. Surprisingly, TP content extracted from Re WL showed a limited dependence on temperature. In particular, TP yield at 200°C (31.40 ± 2.17 mg of GAE/g of dry weight) was only slightly higher than TP extracted at 50°C (21.44 ± 1.59 mg of GAE/g of dry weight).

Water. The use of pure water as solvent drastically reduced the TP yield extraction (Figure 1a). The highest yields were found in Ro and Wh lees at 200°C (21.7 ± 2.60 mg of GAE/g of dry weight and 18.68 ± 1.62 mg of GAE/g of dry weight, respectively). Lower yields were obtained at lower temperatures. Red WL showed an increasing TP yield with increasing temperature from 50 to 100°C (7.82 ± 0.81 and 11.94 ± 1.12 mg of GAE/g of dry weight, respectively). The further increase of temperature until 200°C caused a decrease in TP yields (6.08 ± 0.52 mg of GAE/g of dry weight).

Ethanol. Pure ethanol showed an intermediate behavior, between water and water/ethanol 1/1, in extracting phenolic compounds with MAE process (Figure 1a). A slight increase of TP yield was found increasing temperature from 50 to 150°C. The TP yield increases from 7.13 ± 0.52 mg of GAE/g of dry weight to 26.46 ± 1.76 mg of GAE/g of dry weight for Ro WL and from 6.62 ± 0.61 to 18.78 ± 1.91 mg of GAE/g of dry weight for Wh WL. No significant yield increase was found for TP in Re WL (from 5.40 ± 0.78 to 7.74 ± 1.21 mg of GAE/g of dry weight, $P > 0.05$). A further increase of temperature (from 150 to 200°C) generated an impressive enhancement of TP yield from Ro and Wh WL that reached values of 76.68 ± 6.47 and 66.04 ± 5.98 mg of GAE/g of dry weight. Red WL showed a slight increase in TP yield (21.08 ± 1.81 mg of GAE/g of dry weight) again.

Effect of Na_2CO_3. With the aim to find a new and green way to increase phenols extraction yield, sodium carbonate (2 mmol/g of dry weight of WL) was added to the solvent mixture. Interestingly, higher TP yields were achieved when water added with Na_2CO_3 was used as solvent compared with pure water (Figure 1B). The TP yield from Ro WL reached the highest value at 150°C (61.40 ± 5.64 mg of GAE/g of dry weight). The TP yield from Ro WL without Na_2CO_3 and at the same

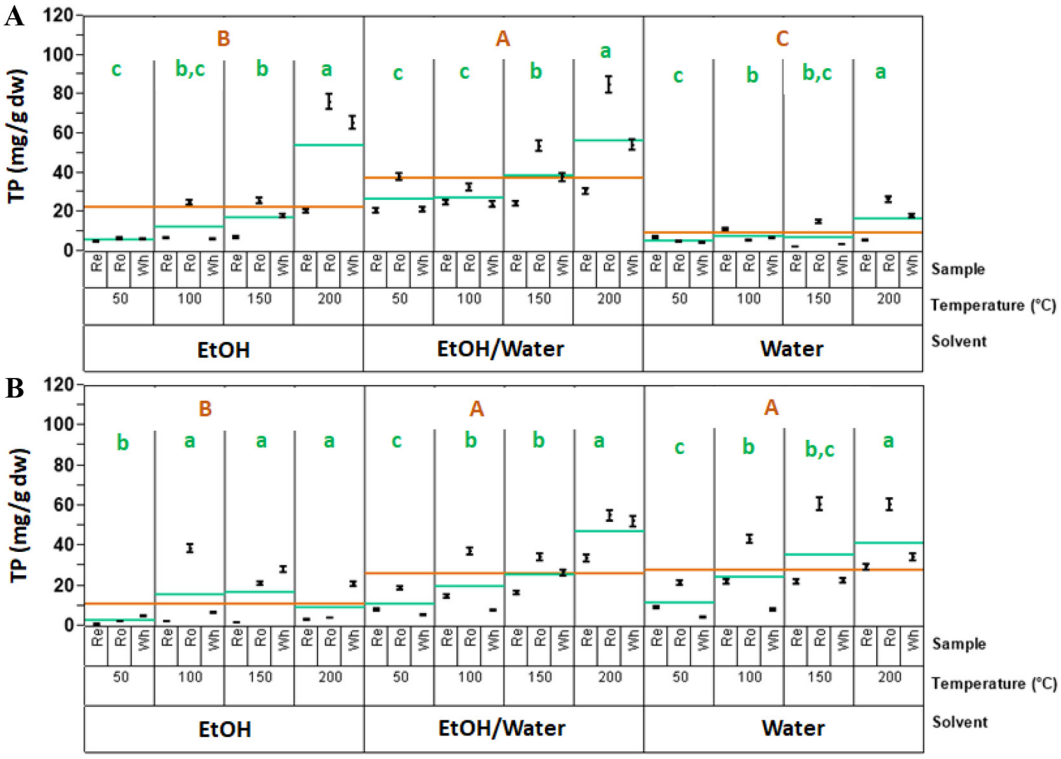

Figure 1. Phenols extraction yields [total phenols (TP); mg/g of dry weight] achieved by microwave-assisted extraction from 3 different wine lees (Wh = white, Ro = rosé, Re = red) at different experimental conditions [solvent: water, ethanol (EtOH)/water 1/1, EtOH; temperature: 50, 100, 150, and 200°C] (A) without Na_2CO_3 and (B) with addition of Na_2CO_3. Black vertical bars = TP means ± SE (n = 3). Brown horizontal lines indicate TP mean values by solvent (water, EtOH/water 1/1, EtOH), n = 36. Brown capital letters indicate significant differences ($P < 0.05$) between TP mean values by solvent. Green horizontal lines indicate TP mean values by solvent (water, EtOH/water 1/1, EtOH) and temperature (50, 100, 150, and 200°C), n = 9. Green lowercase letters indicate significant differences ($P < 0.05$) between TP mean values by solvent and temperature.

temperature was significantly lower (15.64 ± 1.13 mg of GAE/g of dry weight, Figure 1A). An increase was also found for TP from Wh and Re WL that reached the highest values at 200°C (35.04 ± 3.13 and 30.08 ± 2.91 mg of GAE/g of dry weight, respectively).

On the other hand, the use of Na_2CO_3 in the mixture water /ethanol 1/1 seemed to have a negative effect on TP yields that were lower than using water/ethanol 1/1 as solvent without sodium carbonate. This negative effect was further accentuated when ethanol added with Na_2CO_3 was used as a solvent (Figure 1b).

Antioxidant Properties of WL Extracts. The ferric reducing antioxidant power (FRAP) and the ABTS assay for scavenging activity of extracts achieved by MAE from the 3 investigated WL (Wh, Re, and Ro) at different experimental conditions are reported in Figure 2 and Figure 3 (A: without Na_2CO_3; B: with addition of Na_2CO_3).

The multivariate analysis of the overall data set without Na_2CO_3 showed a significant correlation between TP and temperature (r = 0.51, $P < 0.001$), TP and FRAP (r = 0.88, $P < 0.001$), and consequently, FRAP and temperature (0.51, $P < 0.001$; Supplemental Table S1, http://dx.doi.org/10.17632/w4y4k8fh7x.1, Ciliberti et al., 2022). Therefore, the increase in temperature generated an increase in TP extraction. Moreover, FRAP was correlated with phenolic compound concentration. Interestingly, the radical scavenging (ABTS) increased with temperature (r = 0.44, $P < 0.001$), but it was not correlated with TP content, meaning that other compounds, generated from MAE at high temperature, were responsible for this activity (maybe sugar dehydration products or certain subsets of TP). When Na_2CO_3 was used as a catalyst to increase the solubility of phenolic compounds, the positive correlation between TP and temperature (r = 0.51, $P < 0.001$) and FRAP and temperature (r = 0.50, $P < 0.001$) was maintained. However, the correlation between TP and FRAP was lower (r = 0.39, $P < 0.01$). That could be related to the formation of nonphenolic compounds at high temperatures, which had both reducing activity and scavenging activity, as demonstrated by the strong

correlation between FRAP and ABTS (r = 0.73, $P < 0.001$), and ABTS and temperature (r = 0.50, $P < 0.001$).

The multivariate analysis applied to variables (temperature, TP, FRAP, and ABTS) and performed by solvent without Na_2CO_3 (water, water/ethanol, ethanol) showed that the above-described correlations were higher in ethanol (TP vs. temperature, r = 0.73 $P < 0.001$; FRAP vs. temperature, r = 0.72 $P < 0.001$; FRAP vs. phenols, r = 0.90 $P < 0.001$), decreased slightly in ethanol/water 1/1 (TP vs. temperature, r = 0.63 $P < 0.01$; FRAP vs. temperature, r = 0.59 $P < 0.01$; FRAP vs. phenols, r = 0.79 $P < 0.01$), and became not significant in water (Supplemental Table S2, http://dx.doi.org/10.17632/w4y4k8fh7x.1, Ciliberti et al., 2022).

Performing the multivariate analysis between variables (temperature, TP, FRAP, and ABTS) by sample, almost no correlations were found for Red WL (TP vs. FRAP only, r = 0.97, $P < 0.001$), whereas good correlations were found for Ro WL (TP vs. temperature, r = 0.65, $P < 0.01$; FRAP vs. temperature, r = 0.65, $P < 0.01$; FRAP vs. TP, r = 0.90, $P < 0.001$), and Wh WL (TP vs. temperature, r = 0.65, $P < 0.01$; FRAP vs. temperature, r = 0.65, $P < 0.01$; FRAP vs. TP, r = 0.89, $P < 0.001$; ABTS vs. TP, r = 0.74 $P < 0.001$; ABTS vs. FRAP, r = 0.63, $P < 0.001$; Supplemental Table S3, http://dx.doi.org/10.17632/w4y4k8fh7x.1, Ciliberti et al., 2022).

Characteristics of Selected WL Extracts. With the aim to evaluate the potential use as co-formulants in making functional feeds, the bioactivity of microwave extracts from WL was tested on sheep PBMC as an animal model. Six extracts, 2 for each kind of WL investigated, were selected from those tested (Table 2) based on the highest value of total TP or FRAP or ABTS, and obtained at 200°C.

Furthermore, the extracts were subjected to a further liquid-liquid extraction with dichloromethane to better understand the bioactivity of the extracts and its correlation with their chemical composition. The organic fraction was analyzed by chromatography coupled with

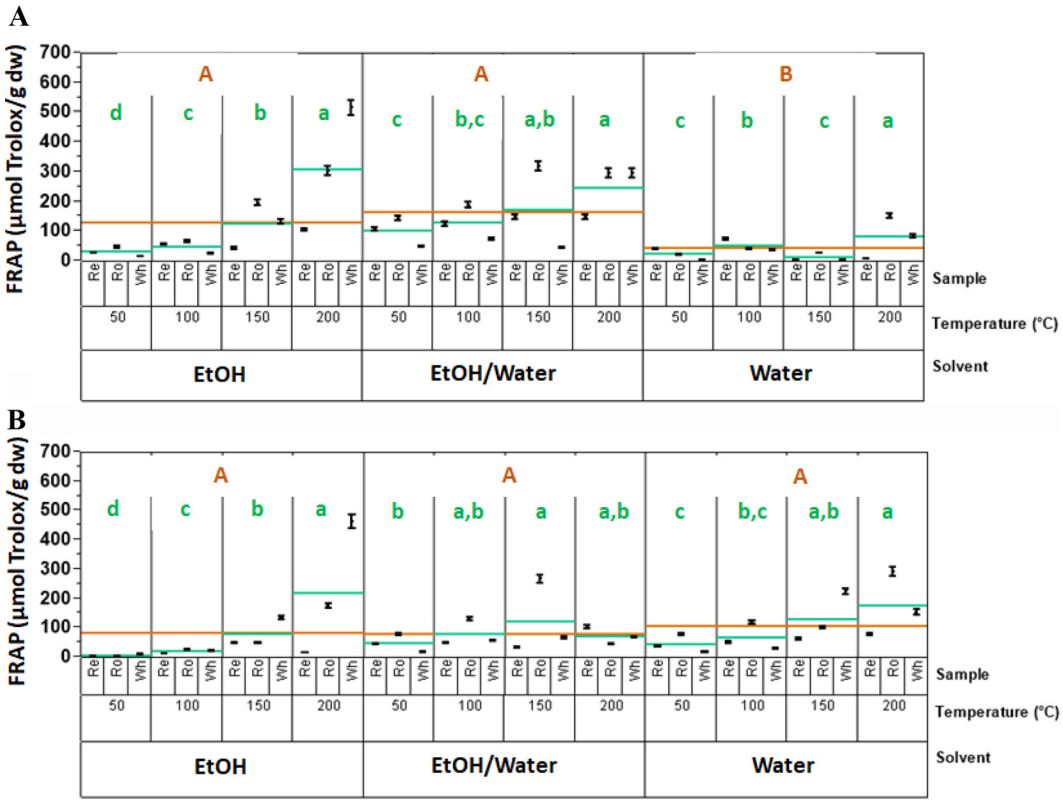

Figure 2. Ferric reducing antioxidant power-ferric antioxidant power (FRAP; µmol of Trolox equivalents antioxidant capacity/g of dry weight) of extracts achieved by microwave-assisted extraction from 3 different wine lees (Wh = white, Ro = rosé, Re = red) at different experimental conditions [solvent: water, ethanol (EtOH)/water 1/1, EtOH; temperature: 50, 100, 150, and 200°C] (A) without Na_2CO_3 and (B) with addition of Na_2CO_3. Black vertical bars = FRAP means ± SE (n = 3). Brown horizontal lines = FRAP mean values by solvent (water, EtOH/water 1/1, EtOH), n = 36. Brown capital letters indicate significant differences ($P < 0.05$) between FRAP mean values by solvent. Green horizontal lines = FRAP mean values by solvent (water, EtOH/water 1/1, EtOH) and temperature (50, 100, 150 and 200°C), n = 9. Green lowercase letters indicate significant differences ($P < 0.05$) between FRAP mean values by solvent and temperature.

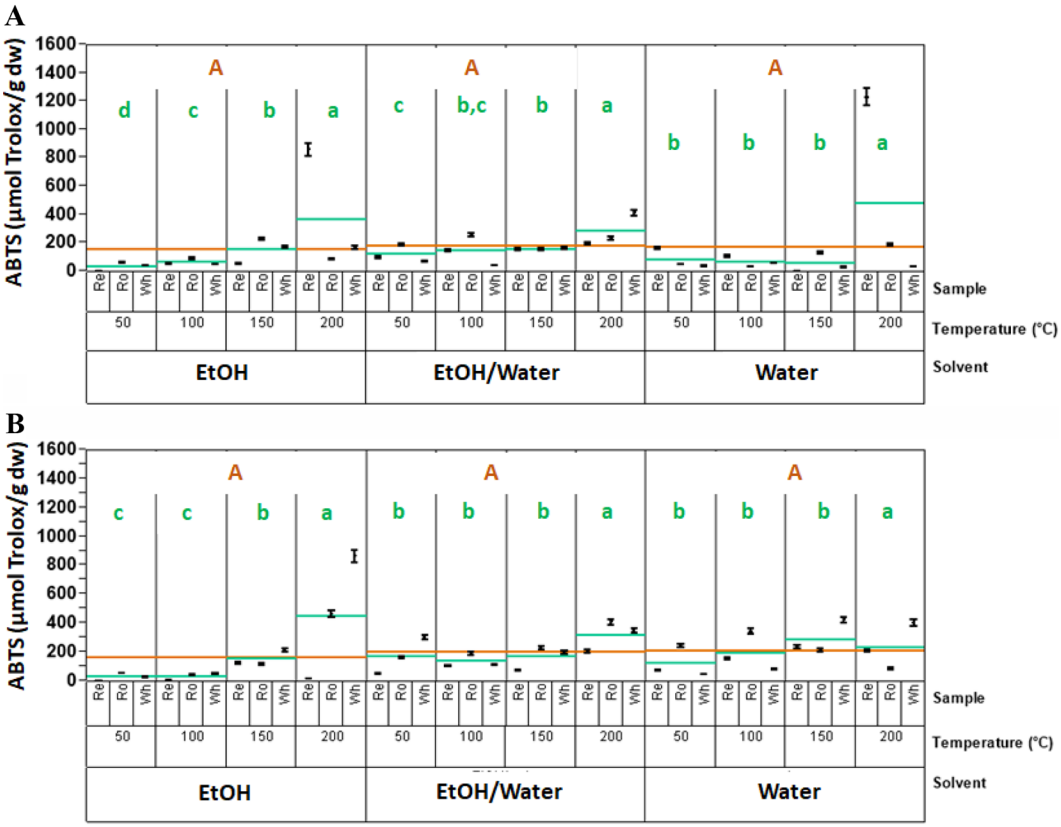

Figure 3. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay, scavenging activity (µmol of Trolox equivalents antioxidant capacity/g of dry weight) of extracts achieved by microwave-assisted extraction from 3 different wine lees (Wh = white, Ro = rosé, Re = red) at different experimental conditions (solvent: water, EtOH/water 1/1, EtOH; temperature: 50, 100, 150, and 200°C; (A) without Na_2CO_3; (B) with addition of Na_2CO_3. Black vertical bars = ABTS means ± standard error (n = 3). Brown horizontal lines = ABTS mean values by solvent (water, EtOH/water 1/1, EtOH), n = 36. Brown capital letters indicate significant differences ($P < 0.05$) between ABTS mean values by solvent. Green horizontal lines = ABTS mean values by solvent (water, EtOH/water 1/1, EtOH) and temperature (50, 100, 150, and 200°C), n = 9. Green lowercase letters indicate significant differences ($P < 0.05$) between ABTS mean values by solvent and temperature.

GC-MS. Figure 4 shows the total ion chromatograms of the 6 selected extracts. Interestingly, in all of them, there were signals that show the presence of diketopiperazines in different quantities and ratios. These compounds, usually achieved by intramolecular cyclization of dipeptides, most probably were generated by cyclization reactions induced by microwaves and high temperatures. Thus, they were not detected in the same extracts obtained at lower temperatures. Diethyl tartrate and diethyl succinate were abundant in Wh

Table 2. Total phenols (TP), reducing power (FRAP), and scavenging activity (ABTS) of selected wine lees extracts to be used for bio-response on sheep animal model

Extract[1]	Temperature (°C)	Solvent	TP (mg of GAE[2]/ g of dry wt ± SE)	FRAP (µmol of trolox/ g of dry wt ± SE)	ABTS (µmol of trolox/ g of dry wt ± SE)
WhEt/k	200	EtOH+Na_2CO_3	21.44 ± 1.03	467.4 ± 35.17	870.6 ± 47.81
WhEtW	200	H_2O:EtOH	54.8 ± 3.75	300.24 ± 22.52	418.56 ± 28.39
RoEt/k	200	EtOH+Na_2CO_3	4.62 ± 0.33	179.2 ± 12.43	474.4 ± 19.23
RoW/k	200	H_2O+Na_2CO_3	61.16 ± 4.82	294.56 ± 28.39	92.68 ± 7.64
ReW	200	H_2O	6.08 ± 0.52	12.36 ± 1.03	1,238.4 ± 64.42
ReW/k	200	H_2O+Na_2CO_3	30.08 ± 2.91	82.8 ± 6.21	215.76 ± 12.73

[1]WhEt/k = wine lees: white; solvent: ethanol plus Na2CO3; WhEtW= wine lees: white; solvent: ethanol and water; RoEt/k= wine lees: rosé; solvent: ethanol plus Na2CO3; RoW/k = wine lees: rosé; solvent: water plus Na2CO3; ReW = wine lees: red; solvent: water; and ReW/k = wine lees: RED; solvent: water plus Na2CO3.

[2]GAE = gallic acid equivalents.

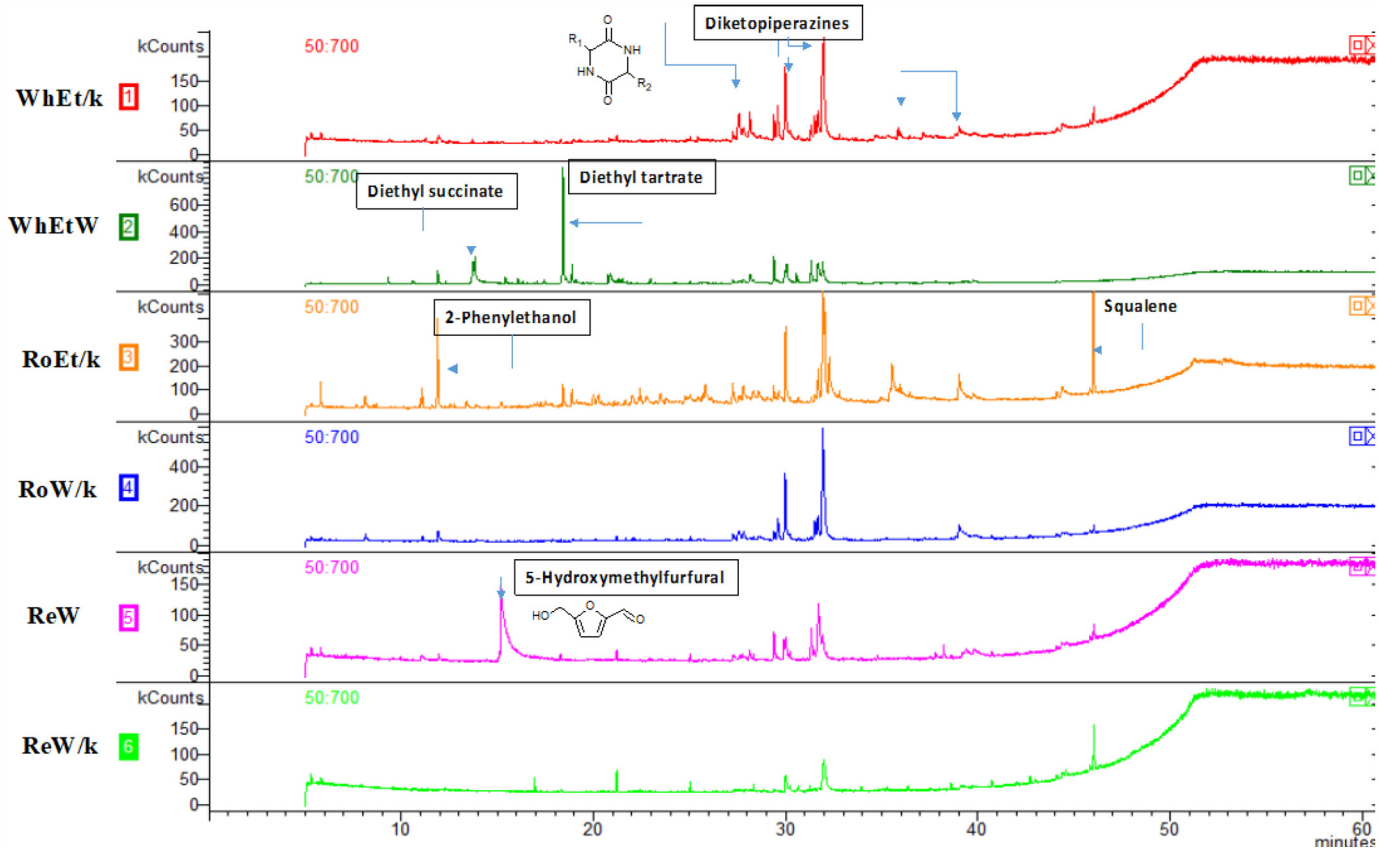

Figure 4. Total ion chromatograms of wine lees extracts that were selected for bioactivity test on animal cells models. WhEt/k: [wine lees: white; solvent: ethanol plus Na_2CO_3; microwave-assisted extraction (MAE); T = 200°C]. WhEtW: (wine lees: white; solvent: ethanol and water; MAE; T = 200°C). RoEt/k: (wine lees: rosé; solvent: ethanol plus Na_2CO_3; MAE; T = 200°C). RoW/k: (wine lees: rosé; solvent: water plus Na_2CO_3; MAE; T = 200°C). ReW: (wine lees: red; solvent: water; MAE; T = 200°C). ReW/k: (wine lees: red; solvent: water plus Na_2CO_3; MAE; T = 200°C).

ethanol/water 1/1 extract, whereas intense signals of squalene and 2-phenylethanol were identified in Ro ethanol plus Na_2CO_3 extract. 5-Hydroxymethylfurfural (5-HMF), an organic compound usually generated by the dehydration of carbohydrates (Francavilla et al., 2016), was also identified with an intense signal only in the Re extract.

Effect of WL Extracts on Cell Proliferation and Apoptosis Proteins Secretion. The WL extracts from Wh (Figure 5A), Ro (Figure 5B), and Re (Figure 5C) winemaking caused a significant antiproliferative effect on PBMC ($P < 0.0001$), showing a decrease of BrdU incorporation in dividing cells similar to PBMC in culture medium.

The level of the pro-apoptotic Bax protein in PBMC supernatants was affected by WL extracts from Wh ($P = 0.03$, Figure 6A), Ro ($P = 0.001$, Figure 6B), and Re ($P = 0.003$, Figure 6C) winemaking. White WL extracts obtained with a combination of water/ethanol and ethanol/Na_2CO_3, used at 0.8 mg/mL, significantly increased the production of pro-apoptotic Bax protein in comparison with levels found in supernatants of both PBMC challenged with LPS and ConA, and PBMC unchallenged. For Re WL extract, obtained with a combination of ethanol/Na_2CO_3 (0.8 mg/mL), an increase of Bax level in comparison with both levels found in supernatants of PBMC challenged with LPS and ConA and PBMC unchallenged was observed. Finally, the Ro WL extracts obtained with a combination of water and Na_2CO_3 showed a higher Bax level at 0.8 mg/mL than supernatant of PBMC challenged with LPS and ConA, PBMC unchallenged and PBMC treated with WL extracts obtained in water extraction. As concern the level of Bcl-2 anti-apoptotic protein, a significant effect was found when PBMC were cultured with WL extracts from Wh ($P = 0.01$, Figure 7A), Ro ($P < 0.0001$, Figure 7B) and Re winemaking ($P = 0.009$, Figure 7C). White WL extract obtained with a combination of ethanol and Na_2CO_3, at 0.8 mg/mL, increased the production of anti-apoptotic Bcl-2 protein significantly in comparison with the levels found in supernatants of PBMC challenged with LPS and ConA and PBMC

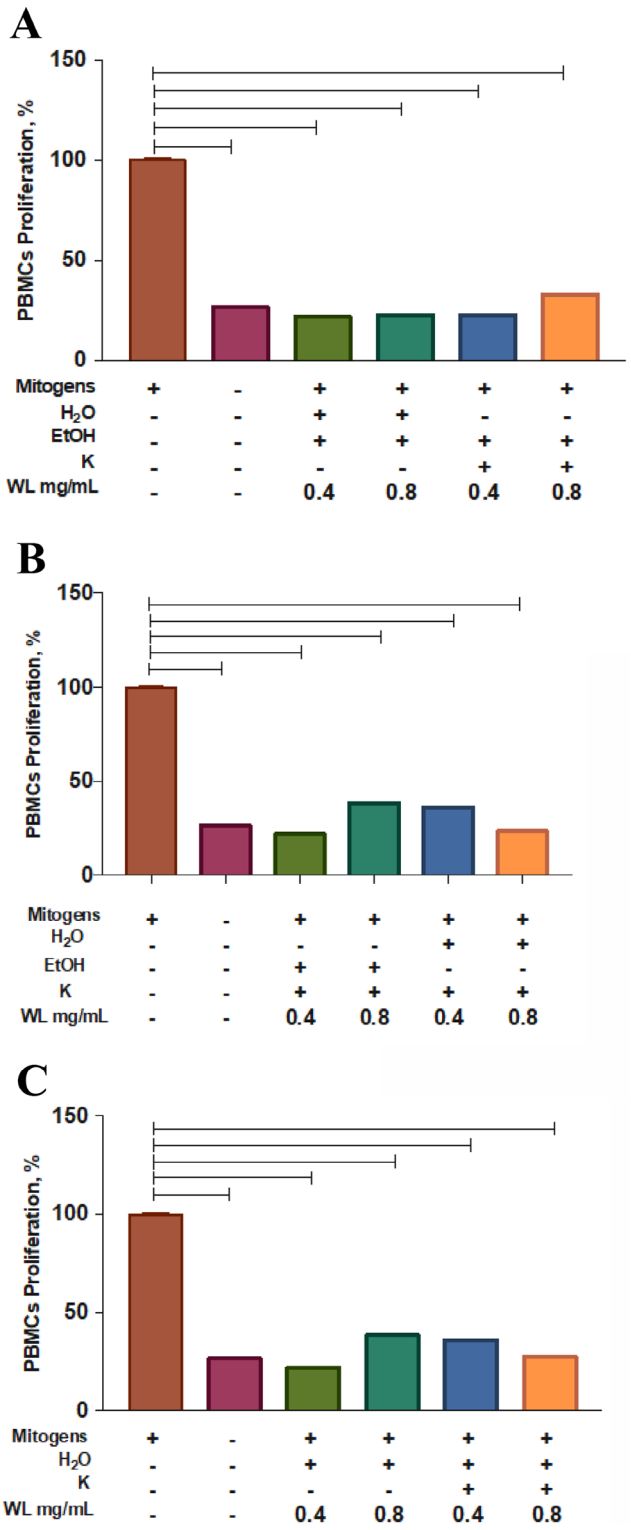

Figure 5. Proliferation of sheep peripheral blood mononuclear cells (PBMC; LSM ± SEM) following in vitro stimulation. The PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with the horizontal lines were considered significant ($P < 0.001$, Tukey test).

unchallenged. For Ro WL extract, obtained with a combination of ethanol/Na_2CO_3 and water/Na_2CO_3 at 0.8 mg/mL, increased the production of anti-apoptotic Bcl-2 protein in comparison with the levels found in supernatants of PBMC challenged with LPS and ConA, PBMC unchallenged and WL extract obtained with a combination of ethanol/catalyzer at 0.4 mg/mL. Finally, the Re WL extract obtained with a combination of water/Na_2CO_3 at 0.8 mg/mL had a higher level of anti-apoptotic Bcl-2 protein in comparison with the levels found in supernatants of PBMC challenged with LPS and ConA, and PBMC unchallenged. To define the role of WL extracts on apoptosis protein production, the ratio of Bax/Bcl-2 was calculated (Figure 8). White ($P = 0.047$, Figure 8A), Ro ($P = 0.016$, Figure 8B), and Re (0.011, Figure 8C) WL extracts were capable of regulating the Bax/Bcl-2 ratio. The Wh WL extract, obtained with a combination of water and ethanol at 0.8 mg/mL, had a higher Bax/Bcl-2 ratio than PBMC challenged with LPS and ConA. A significant increase of the Bax/Bcl-2 ratio was found in Ro WL extract, obtained with a combination of ethanol/Na_2CO_3 at 0.8 mg/mL, in comparison with PBMC challenged with LPS and ConA and PBMC unchallenged. Similarly, the Re WL extracts, obtained with a combination of ethanol/Na_2CO_3 at 0.8 mg/mL, registered an enhancement of the Bax/Bcl-2 ratio compared with PBMC challenged with LPS and ConA ($P = 0.03$) and WL extracts obtained with water at 0.4 mg/mL ($P = 0.03$).

Effect of WL Extracts on Cytokines Secretion. Regards cytokines secreted in PBMC supernatants, IL-6 production (Figure 9A,B,C) was significantly affected by Ro and Re WL extracts ($P = 0.02$, and $P = 0.002$, respectively). The WL extracts, obtained with a combination of water/Na_2CO_3 from Ro winemaking, registered a lower level of IL-6 when WL were tested at a concentration of 0.4 mg/mL than at 0.8 mg/mL. On the other hand, the WL extracts from Re winemaking, obtained with a combination of water/Na_2CO_3 at 0.8 mg/mL, had a higher level of IL-6 than of supernatants of PBMC cultured only with the medium. The levels of IL-1β in PBMC supernatants were depicted in Figure 10. White WL extracts modified the IL-1β secretion, as shown by the lower level registered in supernatants of PBMC treated with WL extracts obtained with a combination of ethanol/water at 0.8 mg/mL than of supernatants of PBMC cultured only with medium ($P = 0.003$, Figure 10A). Moreover, the level of IL-1β found in Wh WL extracts, obtained with a combination of ethanol/water at 0.8 mg/mL concentration, displayed the lowest level of Wh WL extracts obtained with a combination of ethanol/Na_2CO_3, both at 0.4 and 0.8 mg/mL concentration. No significant differences emerged in IL-1β production of PBMC treated with Ro

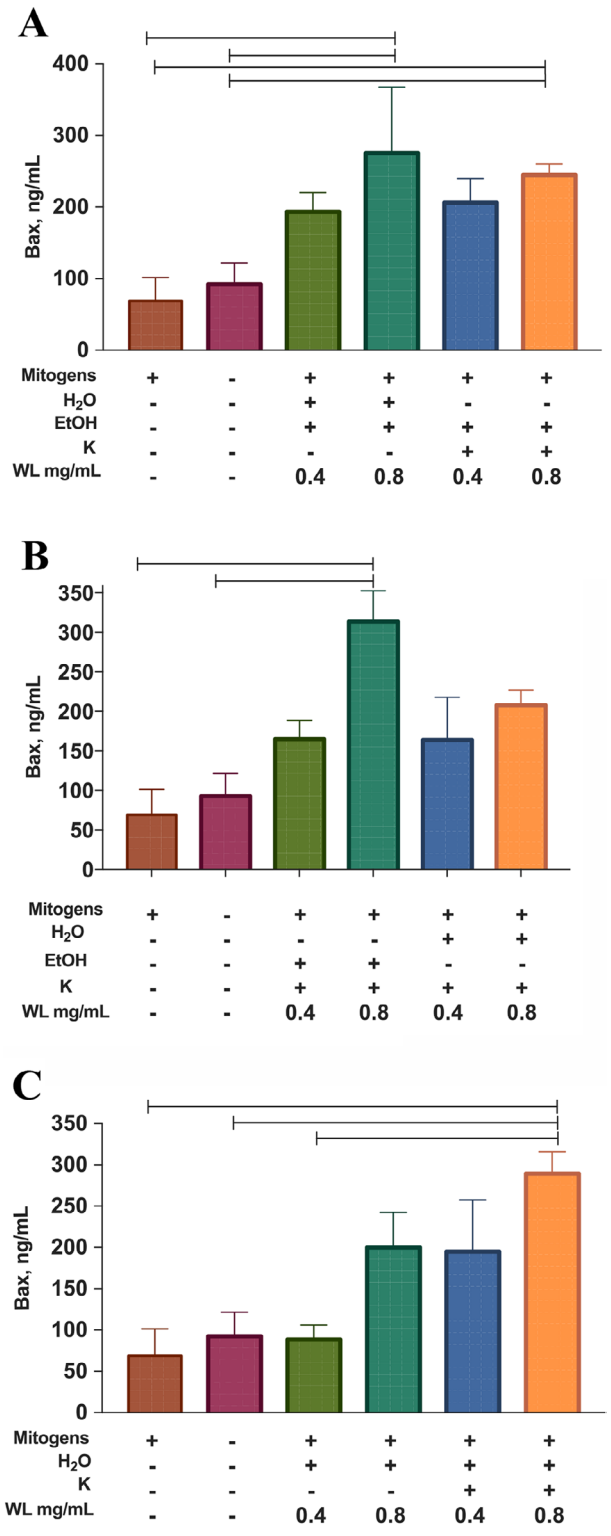

Figure 6. The LSM ± SEM of Bax protein in supernatants of peripheral blood mononuclear cells (PBMC) following in vitro stimulation. The PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with the horizontal lines were considered significant ($P < 0.05$, Tukey test).

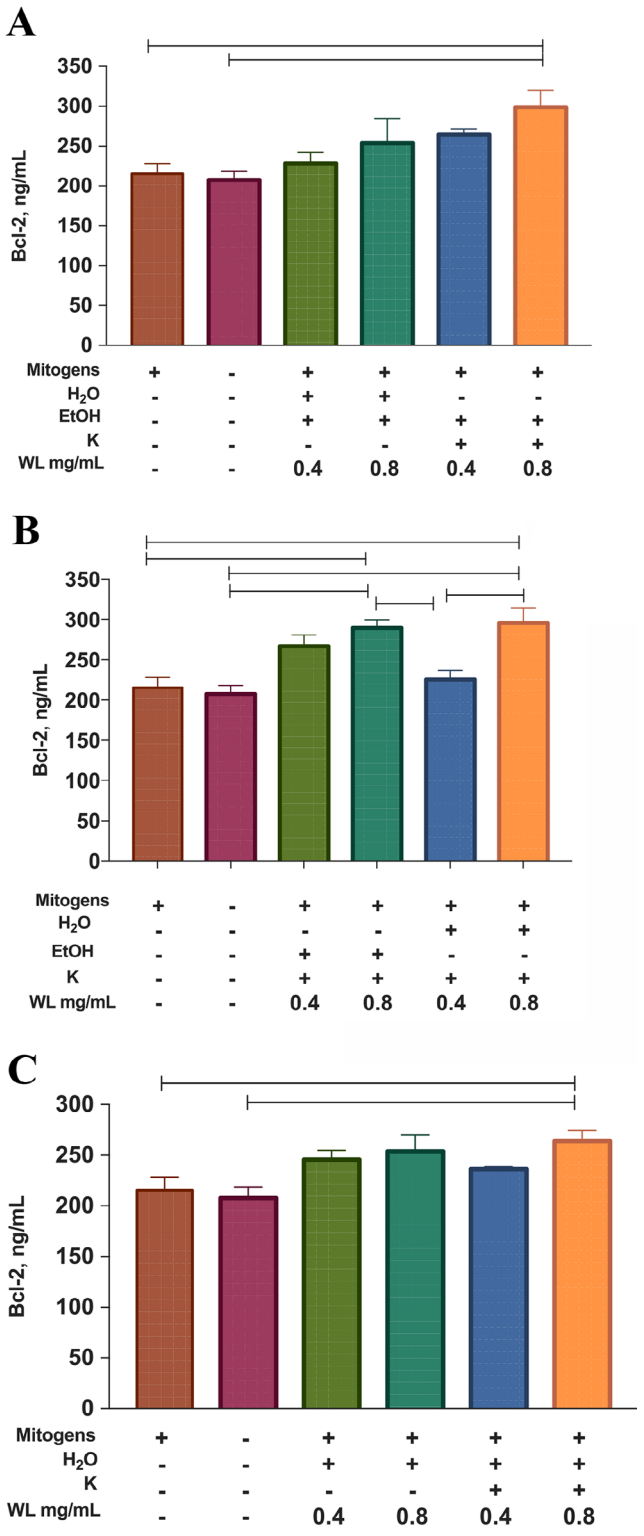

Figure 7. The LSM ± SEM of Bcl-2 protein in supernatants of peripheral blood mononuclear cells (PBMC) following in vitro stimulation. PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with the horizontal lines were considered significant ($P < 0.05$, Tukey test).

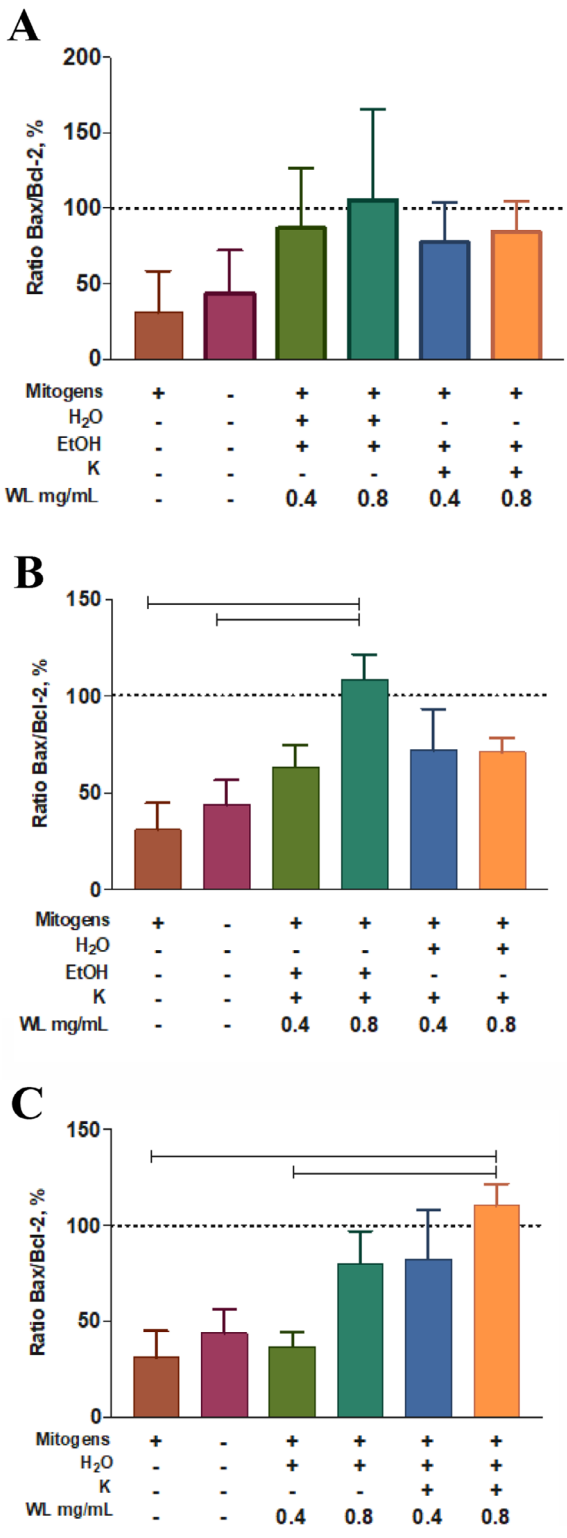

Figure 8. The LSM ± SEM of the ratio Bax/Bcl-2 in supernatants of peripheral blood mononuclear cells (PBMC) following in vitro stimulation. PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with the horizontal lines were considered significant ($P < 0.05$, Tukey test).

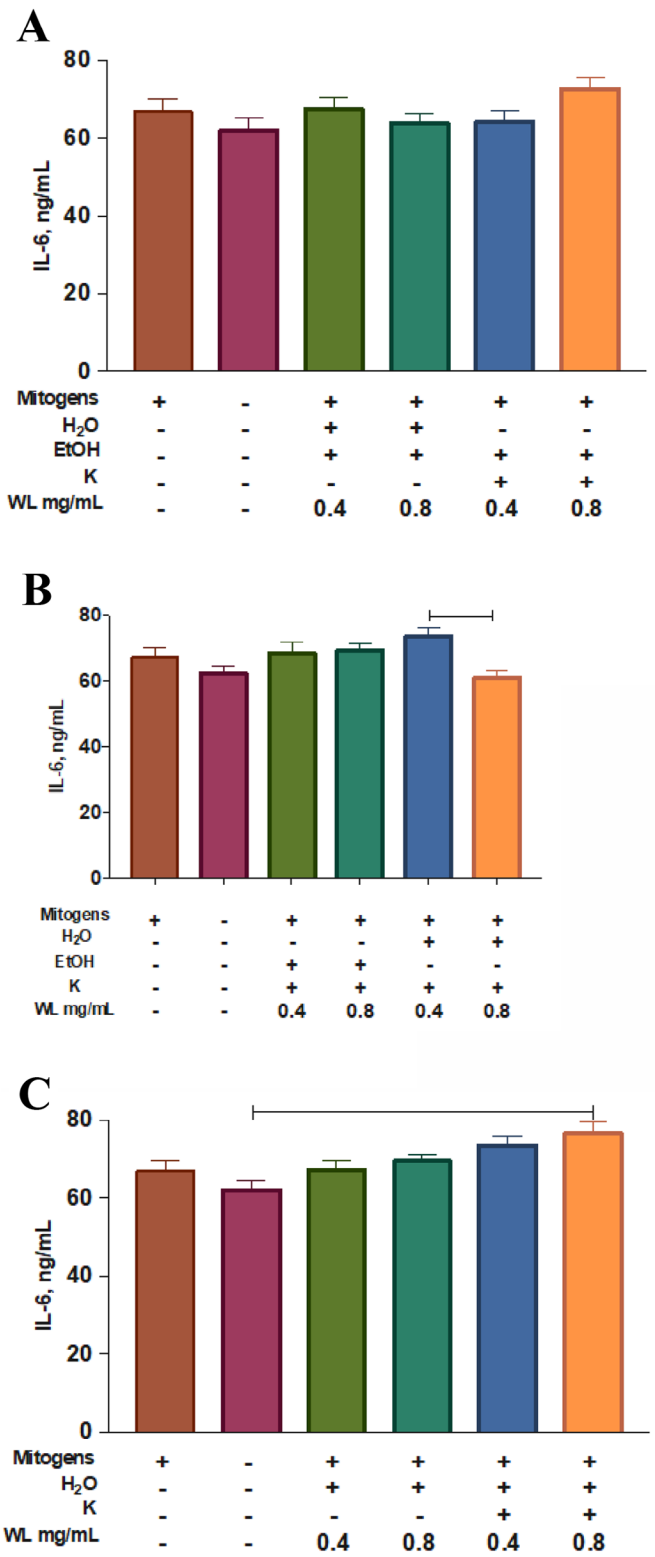

Figure 9. The LSM ± SEM of IL-6 secretion by peripheral blood mononuclear cells (PBMC) following in vitro stimulation. PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with horizonal lines were considered significant ($P < 0.05$, Tukey test).

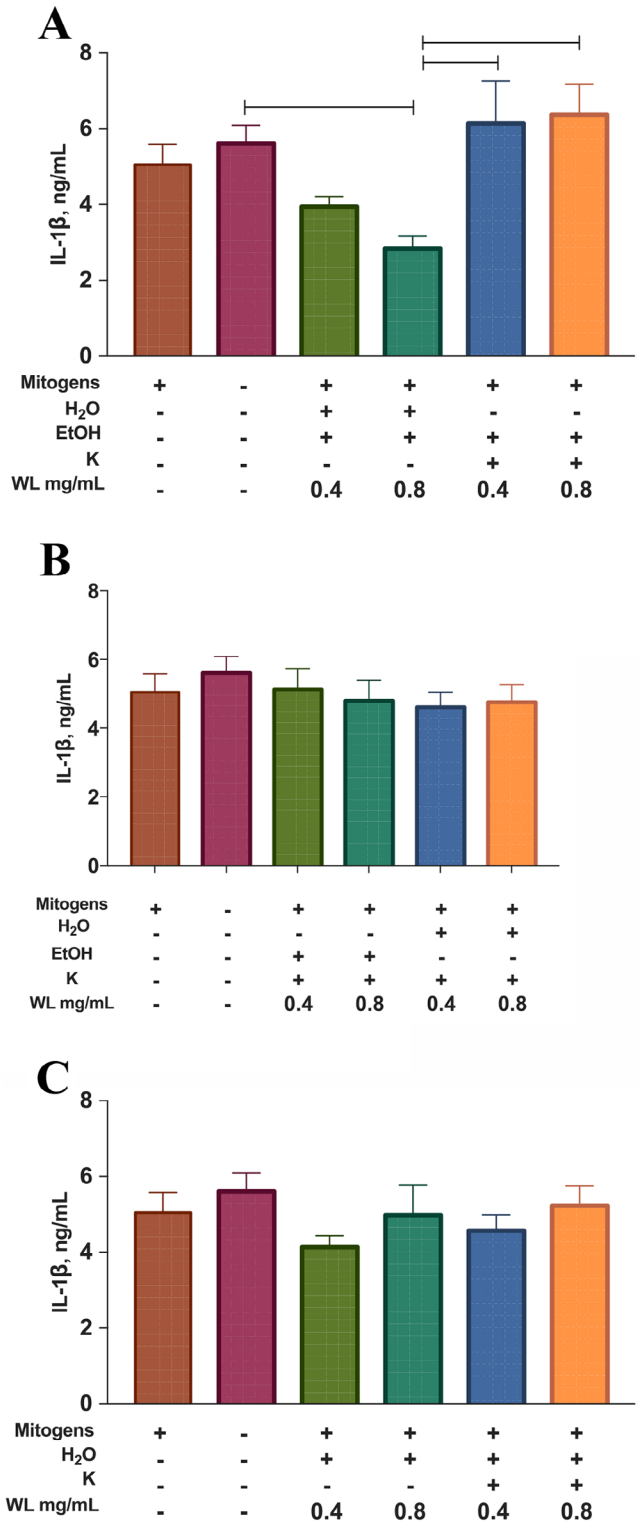

Figure 10. The LSM ± SEM of IL-1β secretion by peripheral blood mononuclear cells (PBMC) following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with horizontal lines were considered significant ($P < 0.05$, Tukey test).

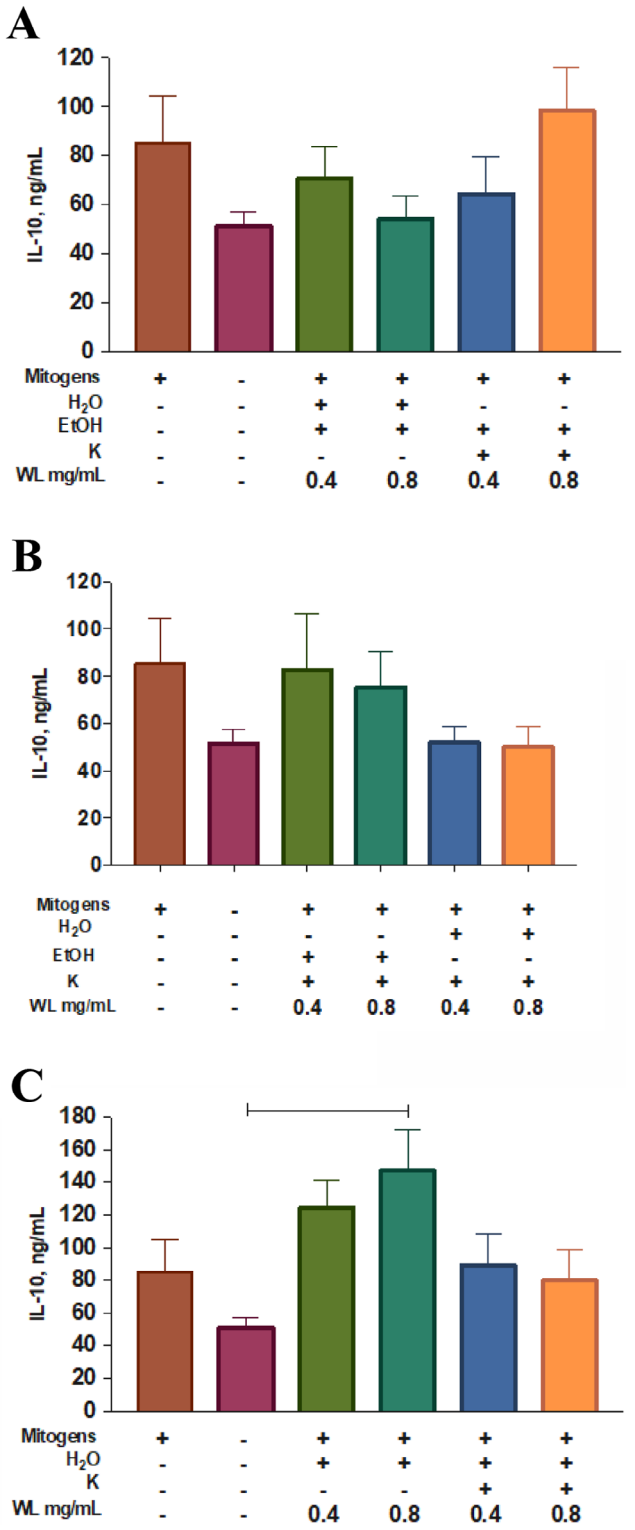

Figure 11. The LSM ± SEM of IL-10 secretion by peripheral blood mononuclear cells (PBMC) following in vitro stimulation. PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with horizontal lines were considered significant ($P < 0.05$, Tukey test).

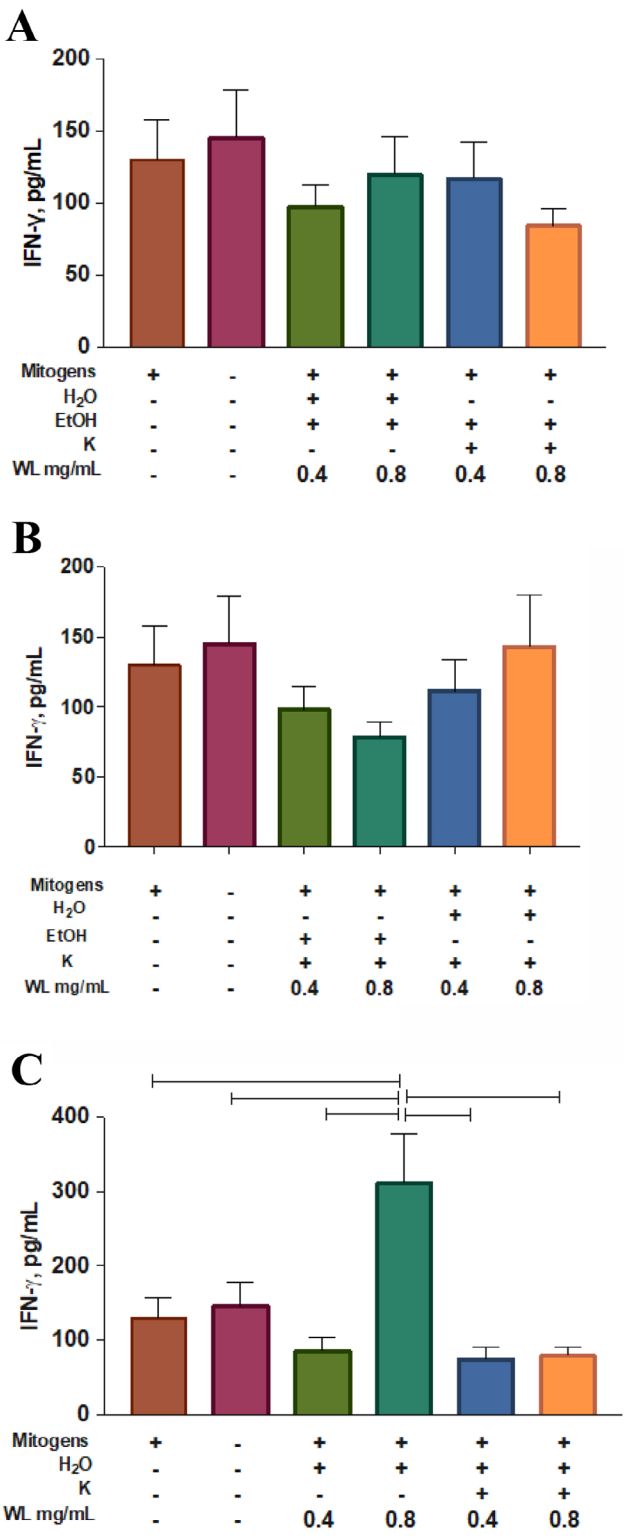

Figure 12. The LSM ± SEM of IFN-γ secretion by peripheral blood mononuclear cells (PBMC) following in vitro stimulation. The PBMC were treated with 2 different concentrations (0.4 and 0.8 mg/mL) of 6 different wine lees (WL) extracts from (A) white, (B) rosé, and (C) red winemaking and in presence of Concanavalin A and LPS. Comparisons with horizontal lines were considered significant ($P < 0.05$, Tukey test).

($P = 0.82$, Figure 10B) and Re WL extracts ($P = 0.48$, Figure 10C). IL-10 secretion of PBMC was not affected by Wh ($P = 0.16$, Figure 11A) and Ro WL ($P = 0.29$, Figure 11B) extracts. On the contrary, the secretion of IL-10 changed in supernatants of PBMC treated with Re WL extracts ($P = 0.009$, Figure 11C). As a result, supernatants from PBMC treated with 0.8 mg/mL of Re WL extracts in water increased the IL-10 production than supernatants of PBMCs unchallenged significantly. Similar data were registered for IFN-γ secretion presented in Figure 12, in which no significant changes when Wh ($P = 0.61$, Figure 12A) and Ro (Figure 12B) WL extracts were used. Whereas the presence of Re WL extracts affected the IFN-γ secretion ($P < 0.0001$, Figure 12C), showing the highest level when WL extracts were obtained with water extraction procedure and at 0.8 mg/mL in comparison to the level of IFN-γ of WL extracts obtained with water/Na_2CO_3 combination and PBMCs challenged with LPS and ConA and PMBC unchallenged.

DISCUSSION

According to the principle of green chemistry and circular economy concept, extraction techniques of by-products, for their evaluation and utilization purpose, should be based on green and suitable technologies that involve the usage of new environmentally friendly and tunable solvents, which can meet both the technological and economic demands (Anastas and Warner 1998). These features allow microwave technology to be applied at different systems to obtain more flexible processes, leading to lower energy consumption and environmental impact (Mellouk et al., 2016). Microwave-assisted extraction of polyphenols from WL have been investigated by Pérez-Serradilla and Luque de Castro (2011) and Arboleda Meija et al. (2019). In both studies, a domestic microwave with adjustable power levels (90–350 W) and irradiation time were used without any temperature control during polyphenols WL extraction. Results on wine lees extraction with solvent showed the central role of ethanol in the extraction process; this is mainly attributed to the higher polarity of water (μ = 1.85 Debye units) compared with the ethanol (μ = 1.69 Debye units) that makes extracted compounds (mainly phenols) less soluble in pure water. In the MAE process, the extraction yield depends on a balanced effect between the solubility of phenols in the solvent, and the energy transfer from the solvent to the matrix, depending on the dielectric constant (ε) of solvent and temperature. Water and ethanol are good microwave absorbers (ε = 87.4 for water and ε = 24.8 for ethanol at 25°C), resulting in the best choice for MAE (Zhou and Liu, 2006). However, when Na_2CO_3 was used as

catalyst, correlations between variables (temperature, TP, FRAP, and ABTS) and type of solvent changed, as demonstrated by multicorrelations found when water was used as a solvent and no correlations found when ethanol was used as a solvent.

The different winemaking techniques that originated the lees (flotation with bentonite for Wh and Ro, and decantation for Re) can influence the phenol content more than grape cultivars. A positive correlation between TP and temperature was found in both WL with bentonite (Wh and Ro); this demonstrated a high concentration and absorption of phenolic compounds on the inorganic substrate (bentonite) that is routinely used for wine fining to reduce, through precipitation and complexation, the excess of phenolic compounds that may increase wine astringency, bitterness, and turbidity (Jiménez-Martínez et al., 2017). The MAE treatment catalyzed the solubilization of organic compounds. However, the increase of temperature up to 200°C can determine depolymerization and degradation of high molecular weight polyphenolic polymers to low molecular weight phenols (Yang et al., 2014; Wang et al., 2019). Moreover, the consequent partial degradation of polyphenolic compounds (i.e., anthocyanins and flavonols) was reported to negatively affect the scavenging activity (ABTS) (Wang et al., 2019) but not the reducing activity as measured by FRAP (Rincón et al., 2019).

Effect of WL Extracts on Cell Proliferation

By-products of wine industry were recently tested as feed on in vivo trials in dairy cows (Santos et al., 2014) and sheep studies (Correddu et al., 2015; Manso et al., 2016). When administrating by-products from grape marc to dairy cows, Moate et al. (2014) found that the dried ingredients, rather than the ensiled, increased milk production, thus suggesting that the physical form of by-products can change their effects on animal production. In the present study, the WL extracts from the 3 different winemaking Wh, Ro, and Re, obtained with MAE coupled with different solvents combination, were tested on in vitro sheep PBMC proliferation, apoptosis proteins, and cytokine secretion. The WL extracts regardless of the 3 different winemaking caused a significant anti-proliferative effect on PBMC.

Wine by-products are considered an interesting source of phenolic compounds, which can exert antioxidant properties in ruminant products (Santos et al., 2014). Red wine phenolic extracts were found to exert multiple anti-inflammatory and anti-atherosclerotic properties on human endothelial inflammatory gene expression (Calabriso et al., 2016). Additionally, grape seed proanthocyanidins can modulate cells immune responses during damage caused by UV radiation, inhibiting the production of proinflammatory cytokines (Katiyar, 2015). However, no data are available on the effect of WL extracts on sheep immune response during in vitro inflammation LPS-mediated. Phytosterols extracted and purified from microalga *D. tertiolecta* were able to suppress sheep PBMC proliferation (Caroprese et al., 2012). It was demonstrated that apoptosis and cell cycle progression are phenomena strictly linked. The programmed cell death is a key negative regulatory event, and its disruption can increase damaged cell accumulation, such as cancer, or inappropriate cell loss, such as neurodegeneration (Hengartner, 1996; White, 1996). One of the most biologically relevant classes of apoptosis regulators acting at the effector stage (Kroemer, 1997; Reed, 1997) of apoptosis, are represented by Bcl-2-related proteins family, among which some members suppress apoptosis, such as anti-apoptotic Bcl-2 protein, and others promote cell death, such as the pro-apoptotic Bax protein. Indeed, the balance between the levels of Bcl-2 and Bax is crucial, especially an excess of Bcl-2 protects cells from apoptosis, otherwise, when Bax is in excess the cells are susceptible to apoptosis. Thus, Bax/Blc2 ratio predetermines the life-or-death response of a cell to an apoptotic stimulus (Oltval et al., 1993; Yang and Korsmeyer, 1996). Our data demonstrated that PBMC treated with WL extracts at a concentration of 0.8 mg/mL showed an increase of apoptosis of Wh, Ro, and Re WL extracts, when obtained with a combination of water/ethanol, ethanol/Na_2CO_3 and water/Na_2CO_3, respectively. On the contrary, Wh WL extract (ethanol/Na_2CO_3), Ro WL extract (water/Na_2CO_3) and Re WL extract (water) did not alter the ratio Bax/Bcl-2 and caused the inhibition of cell proliferation. Similar results were found when Bid, a BH3 domain-only pro-apoptotic molecule that activates the oligomerization of Bak or Bax was studied to elucidate its complex role in apoptosis and survival proliferation. Song et al. (2008) found that etoposide-induced DNA damage of hepatocellular carcinoma cell activated a dual role of Bid, exhibiting S-phase checkpoint activation and a pro-apoptotic role in response to DNA damage in hepatocellular carcinoma cell cells. These suggestions could help to explain the concomitant decrease of cells incorporating BrdU into the DNA, occurring in S-phase of the cell cycle, and the increase of Bax/Bcl-2 ratio, exploiting the potential use of WL extracts as a therapeutic target during cell's DNA damage event.

Effect of WL Extracts on Cytokines Secretion

State of inflammation is a part of homeostasis, named homeostatic inflammation, in which there is

a spontaneous activation of innate immune sensors induced by damage-associated molecular patterns, released from damaged cells, and recognized by cell surface-distributed toll-like receptors (**TLR**), such as TLR1, TLR2, TLR6, or TLR4/MD-2, capable of maintaining a stable immune environment. If the control systems of homeostatic inflammation collapse, then a condition of pathogenic and irreversible inflammation and diseases occurs (Miyake and Fukui, 2016). Phenolic compounds with antioxidant activity can modulate the inflammatory response with suppressive action on pro-inflammatory signaling transduction (Zhang and Tsao, 2016). It has been demonstrated that resveratrol inhibited the (NACHT, LRR, and PYD domains containing protein 3) NLRP3 inflammasome-derived IL-1β secretion in the J774A.1 murine macrophage cell line by preserving mitochondrial integrity and augmenting autophagy (Chang et al., 2015). Accordingly, in the present study, the secretion of IL-1β was reduced in PBMC by Wh WL extract at 0.8 mg/mL. The release of cytokine IL-1β from the cytoplasm into the extracellular environment, after inflammasome activation, activates TRL-1-mediated inflammatory signaling to produce IL-1β, IL-6, IL-8, TNF-α, and IFN-γ. This cascade of signals aims at amplifying the inflammatory response generating a systemic inflammation (Zhang and Tsao, 2016). In the present study, the lower level of IL-1β found after Wh WL extracts treatment did not cause a concomitant change of IL-6 and IFN-γ. On the contrary, Re WL extracted with water and tested at 0.8 mg/mL caused an increase of IFN-γ and IL-10 secretion in PBMC with an activation of immune responses. In vitro studies conducted on several cell lines demonstrated that polyphenols could inhibit the expression and secretion of pro-inflammatory molecules caused by the enhancement of IL-10 production, thus maintaining and regulating the inflammatory:anti-inflammatory ratio (Magrone and Jirillo, 2010; Santangelo et al., 2007). The IFN-γ has a protective action by enhancing the microbicidal function of macrophages as well as cytotoxic functions of T lymphocytes (CD8+ cells) and natural killer (**NK**) cells that respectively have anti-neoplastic (CD8+ and NK cells) and anti-viral functions (CD8+ cells; Kak et al., 2018). High levels of IFN-γ were also registered when phenolic compounds extracted from *Plantago* spp. were tested on human PBMC (Chiang et al., 2003). Moreover, the flavonoid quercetin upregulated the IFN-γ gene expression and production (Nair et al., 2002). The anti-inflammatory IL-10 has different roles, from the inhibition of MHC II (Couper et al., 2008) to the limitation of pro-inflammatory cytokines such as IL-1α and β, IL-6, TNF-α, and chemokines (Mosser and Zhang, 2008). In a previous study on sheep, PBMC treated with a mixture of ergosterol and 7-dehydroporiferasterol extracted from *D. tertiolecta* and challenged with LPS, the production of IL-10 increased dose-dependently (Caroprese et al., 2012). Moreover, the cytokine profile of Re WL extracted in water could be attributed to the high ABTS scavenging activity, ascribed both to the phenol content and to the presence of 5-HMF, which was absent in other extracts. The production of 5-HMF is furfural derived compound produced in food mainly during the second stage of Maillard reaction, when the dehydration and fragmentation of sugar molecules occurs (Tamanna and Mahmood, 2015). It was suggested that 5-HMF has an antioxidant and anti-proliferative action (Zhao et al., 2013), an anti-ischemic effect (Li et al., 2011), protecting the human hepatocyte cell line against damages caused by H_2O_2 (Ding et al., 2010). In addition, in a study conducted by Khodaei and Alizadeh (2017), the ability of 5-HMF to increase the level of IFN-γ by modulating the balance between Th1/Th2 cytokines was demonstrated. During many infections, such as LPS challenge, $CD4^+$ T cells produce both IFN-γ and IL-10 to limit the collateral damage caused by exaggerated inflammation (Trinchieri, 2007). Similarly, the presence of 5-HMF in Red WL extracts obtained with water extraction procedure and tested at 0.8mg/mL can help to explain its modulatory role.

Interleukin-6 is considered a powerful cytokine with pleiotropic effects and recently its role was associated with both pro-inflammatory and anti-inflammatory in a context-dependent manner (Hunter and Jones, 2015). Interleukin-6 promotes the B cells differentiation, T cell activation, and proliferation (Kishimoto, 2006; Dienz and Rincon, 2009; Tanaka et al., 2014). Therefore, the increased level of IL-6 found in PBMC treated with Re WL extract, obtained with a combination of water and Na_2CO_3, could be strictly connected with a high ratio of Bax/Bcl-2. Particularly, it was reported that the increase of IL-6 protects against apoptosis in several tumor cells types by enhancing Bcl-2 production, thus, altering the proliferation and apoptosis balance toward neoplastic cell proliferation; however, a concomitant slight increase of Bax was reported and might be interpreted as an attempt to inhibit cell proliferation (Garcia-Tuñón et al., 2005).

CONCLUSIONS

In the present study, 3 different WL originated by Wh, Ro, and Re winemaking were subjected to microwave-assisted extraction using green solvents and the WL extracts obtained were tested on sheep PBMC. The chemical characteristics of extracts were strictly dependent on WL origins and extraction conditions (solvent, temperature, and Na_2CO_3 presence or

absence). All the WL extracts demonstrated a strong antiproliferative action. The cytokine profile of PBMC treated with WL extract was both dependent on the different WL winemaking origins and the green solvent combination to obtain WL extracts. Data from the present study demonstrate that WL extracts, derived from 3 different winemaking and green solvents combination coupled with MAE, have a bimodal action on control of inflammatory mediated damage. Therefore, the green extraction process (MAE) applied on WL (a by-product of the wine industry) seems to be a promising and challenging route to achieve new valuable bioactive compounds, according to a circular economy approach.

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