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Abstract

Resveratrol is a well-known wine constituent. Its concentration can vary according to the cultivar choice and the winemaking process. Due to its phenolic structure, resveratrol could be transformed under high temperature or oxidative conditions, leading to the formation of various derivatives including oligomers. Hence, the goal of this study is to investigate the presence of these derivatives in wine. In the first stage, hemisynthesis of oligomeric stilbenes was achieved from resveratrol in ethanol by oxidative coupling using metals. Four *de novo* synthetized resveratrol derivatives were identified by MS and NMR spectroscopy including two new molecules, oxistilbenin F and oxistilbenin G. In the second stage, analysis of red wine after heat treatment by LC-MS confirmed the presence of some of these compounds in wine. Finally, the anti-inflammatory effects of the compounds were evaluated by studying their ability to prevent lipopolysaccharide (LPS)-induced upregulation of nitric oxide (NO) and reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line.

Keywords

resveratrol; stilbene; hemisynthesis; wine; oxidative coupling; anti-inflammatory effect

1. Introduction

Polyphenols are among the most valuable compounds found in wine and play a major role in the taste and colour of wine and its potential biological activity (Garrido & Borges, 2013; Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011; Waterhouse, 2002). Most of these compounds such as anthocyanins or tannins are subject to transformations in wines mainly due to oxidative processes influencing the wine properties (Garrido & Borges, 2013). Among them, stilbenes have received great attention. Originally identified in the grapevine and other plants as phytoalexins, (Adrian, Jeandet, Veneau, A. Weston, & Bessis, 1997; Langcake & Pryce, 1977), stilbenes were particularly studied for their several biological effects (Biais et al., 2017; Vang et al., 2011; Zamora-Ros et al., 2008). Resveratrol and its glucoside, the piceid, have the highest concentrations in wine among stilbenes (Guerrero, Valls-Fonayet, Richard, & Cantos-Villar, 2020). Their initial concentrations in grape and wine depend on several biotic and abiotic factors such as environmental stresses, the cultivar, and technological practices (Fernández-Marín, Puertas, Guerrero, García-Parrilla, & Cantos-Villar, 2014; Poussier, Guilloux-Benatier, Torres, Heras, & Adrian, 2003). Their final concentrations in wine are the result of different mechanisms including enzymatic and microbiological interactions, or oxidative reactions. It was demonstrated that piceid can be hydrolysed during fermentation to produce resveratrol, by the yeast β–glucosidases (Roldán, Palacios, Caro, & Pérez, 2010). Resveratrol *cis-trans* isomerization balance can also be altered in wine under light exposure (Mattivi, Reniero, & Korhammer, 1995). In addition, resveratrol can be oxidized in wine kept under heat and oxygen exposure due to its phenolic structure (Bavaresco, Lucini, Busconi, Flamini, & de Rosso, 2016). In fact, oxidative coupling of resveratrol could occur in presence of metallic catalysts like silver, iron or copper in different solvents (Sako, Hosokawa, Ito, & Iinuma, 2004; Velu et al., 2008). The conditions required to induce these reactions can occur naturally in wine, through the presence of ethanol

and several metallic constituents, mainly iron and copper (Płotka-Wasylka, Frankowski, Simeonov, Polkowska, & Namieśnik, 2018; Tariba, 2011). In this study, the oxidative coupling reaction protocol was adapted to obtain new resveratrol dimers in ethanol using silver acetate (AgOAc) and iron chloride ($FeCl_3$), in the aim of investigate the presence of these stilbenes in wine. Compounds were identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) analysis. After identification of the *de novo* produced stilbenes, their content was determined in red wine before and after heat treatment using a liquid chromatography triple quadrupole tandem mass spectrometry (LC-QqQ-MS) method. Finally, their ability to prevent lipopolysaccharide (LPS)-induced upregulation of nitric oxide (NO) and reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line were investigated and compared to that of resveratrol.

2. Material and methods

2.1. Analytical standards and reagents

All reagents were of analytical grade and used as received without further purification. Methanol (MeOH, HPLC grade), ethanol (HPLC grade), acetonitrile (HPLC and LC-MS grades, purity ≥ 99.9%) and formic acid (98%) were purchased from Fisher scientific (Loughborough, United Kingdom). Ethyl acetate (HPLC grade), ferric chloride ($FeCl_3$), Dulbecco's Modified Eagle's Medium (DMEM) and Roswell Park Memorial Institute (RPMI) culture media, foetal bovine serum (FBS), penicillin-streptomycin, lipopolysaccharide (LPS), glutamine, methylthiazolyldiphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Griess reagent, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Sigma-Aldrich (Saint-Louis, USA). *Trans*-resveratrol (3,4',5-trihydroxystilbene, purity ≥ 99%) was supplied by Bulk Powders™ (Colchester, United Kingdom). Silver acetate (AgOAc, purity ≥ 99%) was purchased from Acros organics

(Geel, Belgium). Ultrapure water was obtained from an Elga apparatus (High Wycombe, United Kingdom).

2.2. Oxidative coupling of resveratrol in ethanol

Based on the protocol described by Sako et al. (Sako et al., 2004), regioselective oxidative coupling reaction was first conducted on 600 mg of resveratrol (0.6 g, 12 mmol) using AgOAc (670 mg, 18 mmol) or $FeCl_3$ (2.9 g, 18 mmol) in ethanol (100%, 60 mL) instead of methanol. The reaction mixture was stirred at temperatures ranged between 20 and 50°C. The reaction was then stopped by cooling at 4°C and centrifuged at 4000 rpm for 5 min. Then, the ethanolic fraction was collected and the solvent was removed under reduced pressure using a rotary evaporator. The lyophilization of the fractions obtained from both AgOAc and $FeCl_3$ reactions afforded a dark yellow powder and a light brown powder respectively. Produced compounds from both reactions were isolated and identified using high performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography method with diode array detection (UHPLC-DAD) and NMR.

2.3. Isolation and identification of the reaction products

The preparative HPLC was performed on a Gilson PLC 2050 apparatus (Middleton, WI, USA) equipped with an UV-visible (UV-VIS) detector. Elution was conducted on an Agilent Zorbax SB-C18 column (21.2 mm × 250 mm, 7 µm). The reaction extract powder was solubilized at 50 mg/mL in MeOH-H_2O (50/50; v/v). Extract was then eluted with a flow rate of 20 mL/min using non-acidified ultrapure water (solvent A) and acetonitrile (solvent B), according the following gradient: 35% B (0-2 min), 35-45% B (2-25 min), 45-100% B (25-26 min) and 100% B (26-31 min). The detection was set at 280 and 306 nm and the fractions were automatically collected. The solvent was removed under reduced pressure and fractions

were lyophilized. The elution of the reaction mix produced 3 different fractions, the third one being an isomeric mixture. An additional elution step was then necessary to separate the 2 isomers. The third fraction was eluted with a flow rate of 20 mL/min using non-acidified ultrapure water (solvent A) and methanol (solvent B), according the following gradient: 53% B (0-2 min), 53-56% B (2-25 min), 56-100% B (25-26 min) and 100% B (26-31 min). Compounds purity was measured using an UHPLC-DAD Agilent 1290 series apparatus (Santa Clara, Canada) equipped with an auto sampler module, a binary pump, a degasser, a column heater/selector and an diode array detector (DAD). The elution was performed on an Agilent SB-C18 (2.1 mm x 100 mm, 1.8 µm) column at a flow rate of 0.4 mL/min, with acidified water (0.1% formic acid, solvent A) and acidified acetonitrile (0.1% formic acid, solvent B) according to the following gradient: 10% B (0.0-1.7 min), 10-20% B (1.7-3.4 min), 20-30% B (3.4-5.1 min), 30% B (5.1-6.8 min), 30-35% B (6.8-8.5 min), 35-60% B (8.5-11.9 min), 60-100% B (11.9-15.3 min), 100% B (15.3-17.0 min), 100-10% B (17.0-17.3 min). Samples were injected at a concentration of 100 µg/mL after solubilization in H_2O/MeOH mixture (1/1, v/v). Purity of the isolated compounds was estimated to be greater than 90%. The purified compounds structure was determined by ^{1}H-^{13}C-NMR, using a Bruker Avance III 600 NMR spectrometer (Rheinstetten, Germany). Exact mass was determined by infusion on a Thermo Fischer Scientific Q Exactive Plus Orbitrap (Waltham, Massachusetts, USA) in negative mode with the following parameters: mode full scan; scan range: *m/z* 100-800; resolution: 280k; automatic gain control (AGC) target: $2e^5$; max ion injection: 30 ms. Heated Electrospray Ionization (HESI) source parameters: spray voltage: 2700V; sheath gas flow rate: 8; capillary temperature: 320°C.

2.3.1 Quadrangularin B (2)

Brown amorphous powder. ^{1}H-NMR (600 MHz, methanol-d_4): δ 0.94 (3H, d, J = 7.0 Hz), 2.94 (1H, m), 3.17 (1H, m), 3.32 (1H, dd, J = 3.0, 8.6 Hz), 3.45 (1H, t, J = 3.0 Hz), 3.99 (1H, d, J = 8.6 Hz), 4.27 (1H, d, J = 8.6 Hz), 5.65 (1H, brs), 6.15 (2H, d , J = 1.9 Hz), 6.17 (1H, t, J = 1.9 Hz), 6.23 (1H, d, J = 2.0 Hz), 6.72 (2H, J = 8.5 Hz), 6.75 (2H, d, J = 8.5 Hz) , 6.86 (2H, J = 8.5 Hz), 7.00 (2H, d, J = 8.5 Hz); ^{13}C-NMR (150 MHz, methanol-d_4): δ 14.3, 54.4, 58.3, 60.5, 62.9, 84.1, 100.0, 101.1, 104.7, 105.1, 114.3, 114.4, 122.3, 128.1, 129.1, 131.8, 137.2, 146.1, 150.6, 154.2, 155.4, 156.9, 157.7; HRMS (ESI): *m/z* calcd for $C_{30}H_{27}O_7$ $[M - H]^-$ *m/z* 499.1757, found to be 499.1756.

2.3.2 δ-viniferin (3)

Colorless amorphous powder. ^{1}H-NMR (600 MHz, methanol-d_4): δ 4.45 (1H, d, J = 8.1 Hz), 5.45 (1H, d, J = 8.1 Hz), 6.19 (2H, d, J = 2.1 Hz), 6.24 (1H, t, J = 2.1 Hz), 6.27 (1H, t, J = 2.0 Hz), 6.53 (2H, d, J = 2.0 Hz), 6.85 (2H, d, J = 8.6 Hz), 6.87 (1H, d, J = 8.2 Hz), 6.89 (1H, d, J = 16.3 Hz), 7.05 (1H, d, J = 16.3 Hz), 7.23 (2H, d, J = 8.3 Hz), 7.24 (1H, J = 1.8 Hz), 7.43 (1H, dd, J = 1.8, 8.2 Hz); ^{13}C-NMR (150 MHz, methanol-d_4): δ 57.1, 93.2, 101.6, 101.9, 104.9, 106.6, 109.4, 115.3, 123.1, 126.6, 127.7, 127.9, 128.2 (2C), 131.0, 131.7, 139.8, 144.4, 157.7, 158.8, 159.0, 159.7; HRMS (ESI): *m/z* calcd for $C_{28}H_{21}O_6$ $[M - H]^-$ 453.1338, found to be 453.1343.

2.3.3 Oxistilbenin F (4)

Colourless amorphous powder. ^{1}H and ^{13}C-NMR, see Table 2; HRMS (ESI): *m/z* calcd for $C_{30}H_{27}O_7$ $[M - H]^-$ *m/z* 499.1757, found to be 499.1770.

2.3.4 Oxistilbenin G (5)

Colorless amorphous powder. ^{1}H and ^{13}C-NMR, see Table 2; HRMS (ESI): *m/z* calcd for $C_{30}H_{27}O_7$ $[M - H]^-$ *m/z* 499.1757, found to be 499.1739.

2.4. Wine treatment and stilbene content

2.4.1 Wine treatment protocol

A volume of 50 mL of red wine was heated at 30°C, for 24 hours. Ethanol was eliminated under reduced pressure before proceeding to afford an aqueous wine solution. The samples were then reloaded with pure water to recover a volume of 50 mL to start the solid-phase extraction (SPE) of stilbenes. Hypersep C18 SPE cartridges 5 mg (Thermo Scientific, Rockwood, USA) were previously conditioned with 25 mL of methanol and 25 mL of water. The 50 mL sample was then loaded on the cartridge and a clean-up step with 50 mL of water was conducted. Stilbenes were eluted with 50 mL of methanol and concentrated with a rotatory evaporator to a final volume of 20 mL with 50% methanol.

2.4.2 Stilbene quantification in wine

Stilbenes were controlled and quantified using triple quadrupole-mass spectrometry (QqQ-MS) detection on an Agilent 1260 LC system from Agilent Technologies (Santa Clara, CA, USA). Chromatographic separation was carried out on an Agilent Poroshell C18 (150 mm x 2.1 mm x 2.7 µm) column at 35°C with a binary solvent system composed by acidified water (0.1 % formic acid, solvent A) and acidified acetonitrile (0.1% formic acid, solvent B). The elution was conducted at a flow rate of 0.3 mL/min according to the following gradient: 5-18% B (0-5 min); 18-46% B (5-15 min); 46-95% B (22-25 min); 95% B (25-27 min); 95-5% (27-30 min). The volume of injection for standards and wine samples was 5 µL. The chromatographic system was coupled to an Agilent 6430 Triple Quadrupole mass spectrometer which worked with the following parameters: Drying gas: 11 L/min, Source

187 Temperature: 350°C, Voltage: 3000V. The analysis was performed in optimized multiple
188 reaction monitoring (MRM) conditions obtained by the infusion of pure compounds. For the
189 calibration curve, a solution of 50% methanol containing 10 mg/L of resveratrol,
190 quadrangularin B, δ-viniferin, Oxistilbenin F and Oxistilbenin G was prepared in order to
191 obtain standard solutions by successive 1:2 dilutions.
192
193 **2.5. Biological assays**
194 **2.5.1. Cell culture**
195 Murine macrophage cell line RAW 264.7 was obtained from American Type Culture
196 Collection (ATCC, Rockville, MD, USA). Cells were maintained in DMEM medium
197 supplemented with 10% heat-inactivated foetal bovine serum, 1% streptomycin and 100
198 U/mL penicillin, at 37°C in a humidified, 5% CO_2, 95% air atmosphere.
199 Cells were seeded in 96-well plates (50,000 cells/well). After 24 hours, cells were incubated
200 with increasing concentrations of resveratrol and analogs (1- 20 µM) for 24 hours in the
201 presence or absence of LPS (0.1 µg/mL), in white RPMI medium containing 4 mM of
202 glutamine.
203
204 **2.5.2. Cytotoxicity determination**
205 Cell viability was evaluated by monitoring the cell mitochondrial activity, using the MTT
206 reduction assay. MTT solution (0.5 mg/mL) was added to each well and the cells were
207 incubated for additional 3 hours at 37 °C. Then, the supernatant was removed, formazan
208 crystals were dissolved in DMSO and the absorbance was measured at 595 nm in a
209 spectrophotometer microplate reader (FLUOstarOptima, BMG Labtech).
210
211 **2.5.3. NO measurement**

The nitric oxide (NO) production was determined by measuring the nitrite content in the culture supernatant. Equal volumes of the Griess reagent and the cell culture supernatant were mixed. After 15 min at room temperature, the absorbance was measured at 540 nm (FLUOstarOptima, BMG Labtech). Sodium nitrite standard solution was used to calculate NO concentration.

2.5.4. Intracellular ROS measurement

Generation of and reactive oxygen species (ROS) in cells was analysed using a fluorometric probe, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). After treatment, medium was removed, cells were washed with phosphate-buffered saline (PBS) and incubated in the presence of 5 µM H_2DCFDA at 37°C for 30 min. Then, the fluorescence intensity was quantified using a spectrofluorometer (FLUOstarOptima, BMG Labtech). The wavelengths of excitation and emission used to detect the ROS were 485 nm and 520 nm, respectively.

2.6. Statistical analysis

Experiments were performed in quadruplicate and repeated at least three times. All values were represented as mean ± standard error of the mean (SEM). Data were analysed by one-way analysis of variance followed by a Dunnett's post-test using GraphPad Prism Software (Sa Diego, CA, USA). Significance was set at $p < 0.05$.

3. Results and discussion

3.1. Treatment of resveratrol by metals

Resveratrol was mixed with silver acetate (AgOAc) or iron chloride ($FeCl_3$) in ethanol followed by chromatographic purification using preparative HPLC and structure identification by HRMS and NMR spectroscopy. The reaction between resveratrol and metals in ethanol led

to the formation of four main compounds: quadrangularin B (**2**), δ-viniferin (**3**), oxistilbenin F (**4**), and oxistilbenin G (**5**), as shown in Table 1 and Figure 1.

Compound **2** was identified as quadrangularin B (Adesanya et al., 1999). The relative stereochemistry of quadrangularin B (**2**) was supported by the examination of ROESY spectrum. The stereochemistry of C-7b and C-8b was established by the presence of NOE correlations H-7b/H-10b and H-8b/H-2b. The correlations H-8a/H-10b and H-8a/H-7b indicated that H-8a and H-7b are one the same side. Finally, NOE cross-peaks H-2a/H-14a and H-2b/H-1 indicated the stereochemistry of C-1 (Adesanya et al., 1999). Compound **3** was the main compound formed (yield 48% in AcOAg, after 1 h at 50°C). The structure of compound **3** was identified as δ-viniferin by comparison with previously reported mass and NMR data (Pezet et al., 2003). The ^{1}H-NMR spectrum of δ-viniferin (**3**) exhibited the characteristic signals for the dihydrobenzofuran ring at δ 5.45 (d, J = 8.1 Hz) and 4.45 (d, J = 8.1 Hz), and for the *trans* vinyl protons at δ 7.05 (d, J = 16.3 Hz) and 6.89 (d, J = 16.3 Hz). Compound **4** was obtained as an amorphous brown powder, with a high-resolution molecular ion in negative mode at *m/z* $[M - H]^-$ 499.1770 (calcd for $C_{30}H_{27}O_7$ *m/z* 499.1757) corresponding to a resveratrol dimer. The ^{1}H-NMR spectrum of compound **4** (Table 2) exhibited two AB_2 systems at δ 6.14 (1H, brs, H-12a), 6.41 (2H, d, J = 2.1 Hz, H-10a/14a), and δ 6.14 (1H, brs, H-12b), 6,26 (2H, d, J = 2.1 Hz, H-10b/14b) for rings A_2 and B_2, respectively; two AA'XX' type ortho-coupled aromatic protons at δ 7.28 and 6.72 (2H each, d, J = 8.6 Hz) for ring A_1, δ 6.72 and 7.12 (2H each, d, J = 8.7 Hz) for ring B_1; two *trans* coupled olefinic bond proton signals at δ 6.77 (1H, d, J = 16.1 Hz, H-8a) and 6.90 (1H, d, J = 16.1Hz, H-7a), two coupled aliphatic protons at δ 4.44 (1H, d, J = 5.8 Hz, H-7b) and 5.10 (1H, d, J = 5.8 Hz, H-8b), and one ethoxy group at δ 1.08 (3H, J = 7.0 Hz), 3.28 (1H, m) and 3.42 (1H, m). The correlations of the aromatic rings, double bond, aliphatic protons were deduced from COSY spectrum. All the protonated carbons were identified from HSQC

spectrum. The examination of the C-H long range correlations from HMBC spectrum indicated that compound **4** was a chain resveratrol dimer connected with C-8b and OH-4a. This connectivity was confirmed by the HMBC cross-peaks C-3a/H-8b and C-8b/H10b. The position of the ethoxy group was deduced from the HMBC cross-peak between OCH_2 and H-7b. Concerning relative stereochemistry, the absence of strong NOE correlations H-8b/H-2b and H-7b/H10b indicated that C-7b and C-8b should be in *threo* form. The structure of the new compound **4** was named oxistilbenin F. Compound **5** was obtained as a brown amorphous powder, with a high-resolution molecular ion in negative mode at *m/z* $[M - H]^-$ 499.1739 (calcd for $C_{30}H_{27}O_7$ *m/z* 499.1757). The NMR data (Table 2) of **5** were nearly identical to those of oxistilbenin F (**4**). Examination of 2D NMR spectra suggested that compounds **4** and **5** are a pair diastereoisomers. In contrast to ROESY spectrum of oxistilbenin F (**4**), the strong NOE cross-peaks H-8b/H-2b and H-7b/H10b observed for compound **5** suggested that C-7b and C-8b should be in *erythro* form. In addition, the large and small values for H-7b and H-8b measured for *erytrhro* and *threo* forms, respectively, were in agreement with thus observed in close diastereoisomers (Li et al., 2017). The structure of the new compound **5** was named oxistilbenin G.

As previously observed, metals induce resveratrol dimerization by oxidative coupling (Sako et al., 2004; Velu et al., 2008). The reaction can be explained by a single electron transfer from resveratrol to metal cation followed by a regioselective coupling and intramolecular cyclization. The dimerization process is mainly controlled by the one-electron oxidant and the solvent used (Sako et al., 2004; Velu et al., 2008). The efficiency of the reaction (Table 1) depends of the oxidation ability of the employed oxidative reagents and temperature. In all cases, δ-viniferin (**3**) was the major product and quadrangularin B (**2**) and oxistilbenin F (**4**) were obtained as minor products. Comparing the metallic catalysts used, the reaction is much more effective with silver acetate (total yield 87%, after 1 hour at 50°C) than with $FeCl_3$ (total

yield 18%, after 1 hour at 50°C). When $FeCl_3$ was employed, quadrangularin B (**2**) and oxistilbenin F (**4**) were hardly produced, while δ-viniferin (**3**) and oxistilbenin G (**5**) were formed in lower quantities. The mechanism of formation of the different dimers could be similar to that reported for isorhapontigenin (Wang et al., 2014). In fact, the oxidative reaction on OH-4 position could induce the formation of phenoxy radicals leading large diversity of coupling modes. Quadrangularin B (**2**) was previously obtained by biomimic transformation of resveratrol using peroxidases (Takaya et al., 2005). Oxistilbenin F (**4**) and G (**5**) were synthetized and reported for the first time.

3.2. Formation of resveratrol dimers in wine after heat treatment

Resveratrol is one of the main stilbenes identified in wines. Even if its amount vary greatly, its average content is 2.7 mg/L in red wines (Neveu et al., 2010). In addition, oxidative reagents, such iron, are naturally present in wine (Tariba, 2011). Based on the results obtained in this work, resveratrol dimerization could occur in wine. To confirm this hypothesis, a red wine was subjected to a heat treatment at 30°C for 24 hours. Stilbenes were quantified before and after wine heating using a LC-MS method on a triple quadrupole mass spectrometer (LC-QqQ-MS). MRM mode was used to select specific transitions of the quantified compounds in order to increase both selectivity and sensitivity (Lambert et al., 2015). For each compound, MRM transitions parameters were optimised. The optimised MRM transition parameters are shown in Table 3. For quantitative analysis, the quantifier transition chromatogram of stilbene was used, and the rest of transitions confirmed the stilbene identification in wine. The quantifier transitions were *m/z* 227/143 for resveratrol (**1**), *m/z* 499/359 for quadrangularin B (**2**), *m/z* 453/361 for δ-viniferin (**3**), *m/z* 499/227 for oxistilbenin F (**4**), and *m/z* 499/453 for oxistilbenin G (**5**).

311 The amounts of the five quantified stilbenes before and after wine treatment were reported in
312 Table 4. Before wine treatment, the level of resveratrol was found to be 330 µg/L in the red
313 wine. In addition, a small amount of δ-viniferin was observed (20 µg/L). This compound is a
314 natural stilbene previously identified in red wine (Vitrac et al., 2005). After wine treatment
315 for 24 hours at 30°C, the level of resveratrol had decreased by almost 50%, while the amount
316 of δ-viniferin had increased by 5-fold bringing it from 20 to 100 µg/L. In addition, the
317 formation of oxistilbenin G was observed in red wine after the heat treatment. This compound
318 was specifically formed after oxidative coupling of two resveratrol units in ethanol. The
319 δ-viniferin was the main compound formed in red wine followed by oxistilbenin G, in
320 agreement with the rate observed in the reaction between resveratrol and metals in ethanol
321 (Table 1). All these data have confirmed that resveratrol could be transformed by oxidative
322 coupling during wine aging providing oligomers. The difference between the total stilbene
323 content before and after treatment (1.50 and 0.94 µM, respectively) seems to indicate that a
324 part of the resveratrol reacted with other compounds.
325
326 **3.3. Anti-inflammatory activity of resveratrol derivatives**
327 Anti-inflammatory activity of the produced compounds was evaluated using LPS-induce
328 RAW 264.7 cells model. In order to determine the cytotoxicity of stilbenes, the viability was
329 measured after cells treatment at 1 to 20 µM. Resveratrol and quadrangularine B did not show
330 significant cytotoxicity at all tested concentrations, whereas δ-viniferin, oxistilbenin F and
331 oxistilbenin G exhibited cellular toxicity at 15 µM (data not shown). On one hand, as shown
332 in the Fig. 2A, resveratrol, δ-viniferin, oxistilbenin F and oxistilbenin G reduced NO
333 production by LPS-stimulated macrophages, with IC_{50} of 17.9, 8.7, 9.2 and 9.8 µM,
334 respectively. On the other hand, quadrangularin B exhibited a moderate inhibitory activity
335 against NO at the highest concentration. In addition, a decrease effect on the production of

ROS was observed for all stilbenes except the quadrangularin B (Fig. 2B). The δ-viniferin was the most effective (IC_{50} 7.6 µM). Thus, oxidative coupling during wine aging could modulate the biological activities associated with red wine stilbenes. This study confirms that red wine storage temperature is an important consideration to preserve wine properties.

4. Conclusion

In this study, we demonstrated that oxidative dimerization of resveratrol could occur in red wine after heat treatment. Heating the wine modulates the stilbene content by reducing the resveratrol amount, increasing the amount of δ-viniferin, and inducing the formation of new compounds such as oxistilbenin G. Finally, the anti-inflammatory activity of these compounds was evaluated. The results have indicated that oxidative coupling during wine aging could modulate the biological properties of wine by inducing the formation of more active compounds such δ-viniferin.

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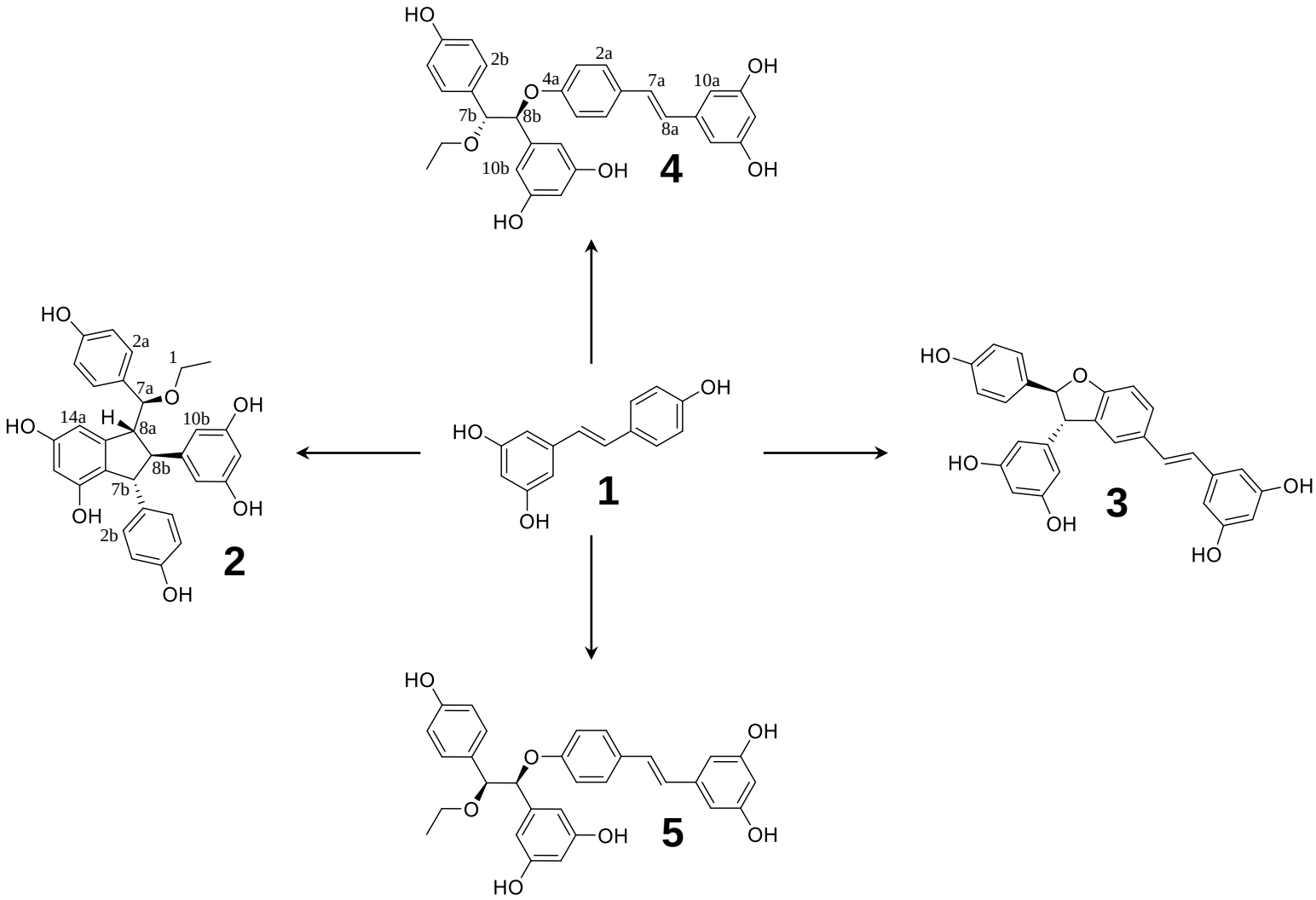
439 **Figure legends**

440 **Figure 1.** Structures of the compounds formed after oxidative coupling of resveratrol.

441

442 **Figure 2.** Effect of treatment with stilbene and LPS (0.1 µg/mL) on the NO production (A) or
443 ROS formation (B) in RAW 264.7 cells. Res = resveratrol, δ-vin = δ-viniferin, Oxi F =
444 oxistilbenin F, Oxi G = oxistilbenin G and Qua B = quadrangularin B. Data are expressed as
445 percentage of the control (cells treated with LPS alone set to 100% production),
446 corresponding to the mean ± SEM (n=4).

447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2a
4a
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OH
O
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8b
8a
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OH
OH
HO
4
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1
7a
O
14a
H
8a
10b
OH
HO
8b
7b
OH
OH
2b
OH
2
HO
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1
HO
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OH
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OH
HO
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2b
OH
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O
OH
OH
HO
5

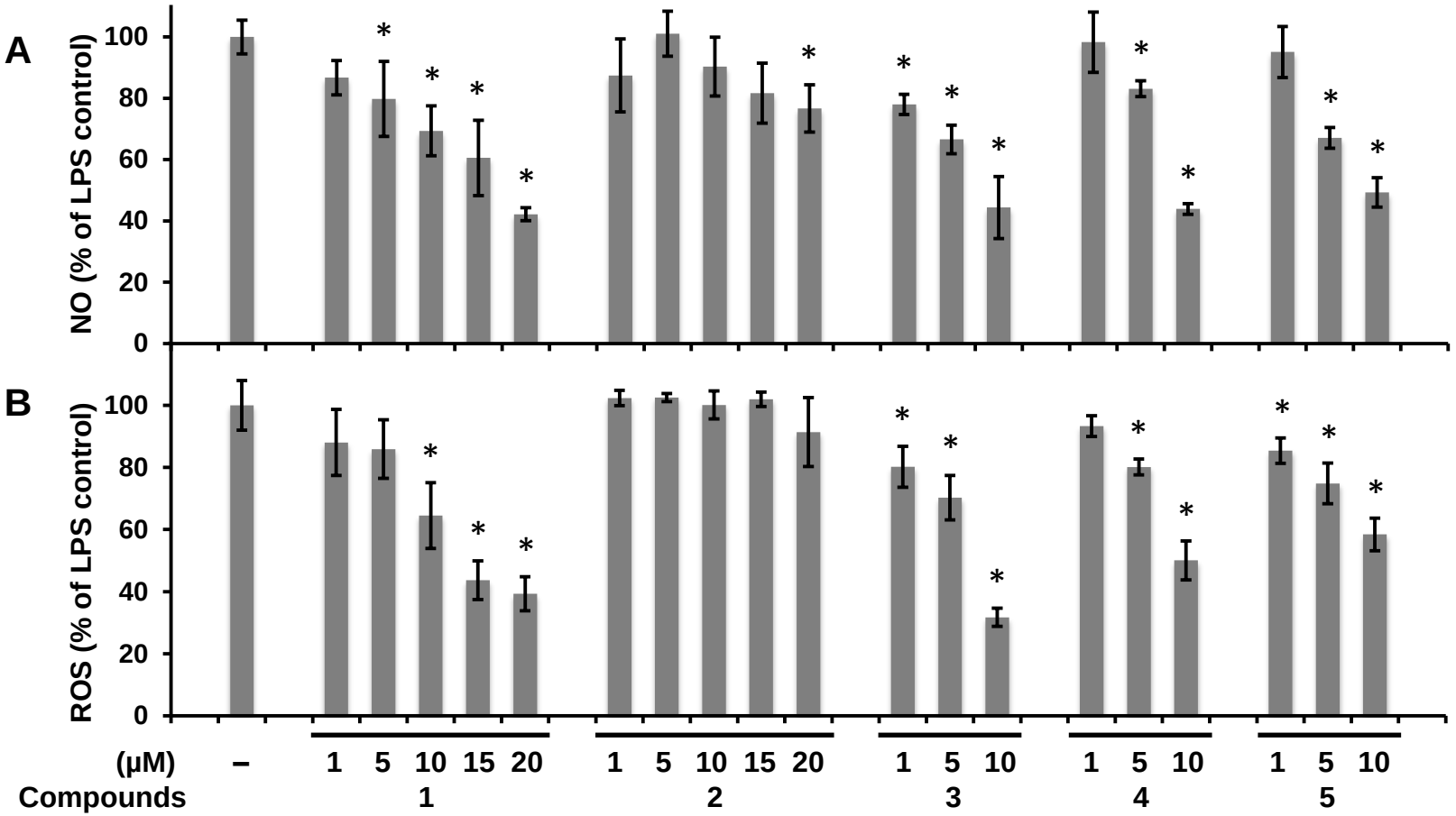

A
NO (% of LPS control)
0
20
40
60
80
100
B
ROS (% of LPS control)
0
20
40
60
80
100
(μM)
–
1 5 10 15 20
1 5 10 15 20
1 5 10
1 5 10
1 5 10
Compounds
1
2
3
4
5

Table 1. Treatments of resveratrol (**1**) with different oxidizing reagents in ethanol.

Reagent	**Temperature (°C)**	**Time (h)**	**Yield (%)**			
			2	**3**	**4**	**5**
AgOAc	50	1	3	48	6	32
AgOAc	40	1	2	29	4	19
AgOAc	40	2	2	33	5	22
AgOAc	30	1	0	15	2	10
AgOAc	20	1	0	3	0	0
$FeCl_3$	50	1	1	10	1	6

Table 2. NMR data of compounds **4** and **5** in methanol-d_4.

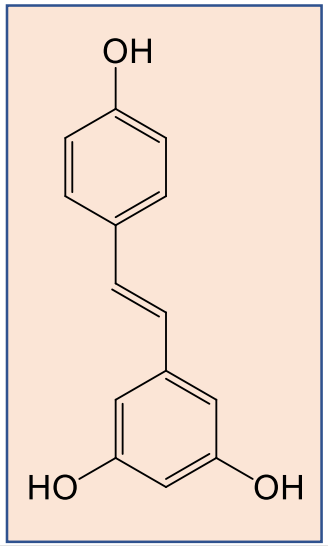
	4		**5**	
	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1a	130.2	-	130.2	-
2a/6a	127.1	7.28 (d, 8.7)	127.1	7.33 (d, 8.7)
3a/5a	115.1	6.72 (d, 8.7)	115.2	6.85 (d, 8.7)
4a	157.9	-	158.1	-
7a	127.8	6.90 (d, 16.1)	127.8	6.92 (d, 16.1)
8a	126.6	4.24 (d, 16.1)	126.6	6.79 (d, 16.1)
9a	139.7	-	139.7	-
10a/14a	104.6	6.41 (d, 2.1)	104.6	6.42 (d, 2.1)
11a/13a	158.2	-	158.3	-
12a	101.4	6.14 (brs)	101.4	6.14 (t, 2.1)
1b	129.2	-	128.8	-
2b/6b	129.2	7.12 (d, 8.7)	128.8	6.99 (d, 8.7)
3b/5b	114.3	6.72 (d, 8.7)	114.2	6.66 (d, 8.7)
4b	156.7	-	156.7	-
7b	84.7	4.44 (d, 5.8)	85.2	4.52 (d, 6.9)
8b	82.7	5.10 (d, 5.8)	83.9	5.05 (d, 6.9)
9b	141.4	-	140.6	-
10b/14b	105.8	6.26 (d, 2.1)	106.0	6.11 (d, 2.1)
11b/13b	157.8	-	157.8	-
12b	101.4	6.14 (brs)	101.4	6.05 (t, 2.1)
OCH_2	64.6	3.28 (m)	64.6	3.46 (m)
		3.42 (m)		
CH_3	14.1	1.08 (t, 7.0)	14.1	1.17 (t, 7.0)

Table 3. MRM parameters (precursor and product ions *m/z*, retention times), and quantitative response of stilbene compounds in wine.

Compound	**Formula**	**Mode**	**Precursor ion** ***m/z***	**Quantifier** ***m/z***	**Qualifiers** ***m/z***	**Retention time (min.)**	**Calibration range (mg/L)**
resveratrol (**1**)	$C_{14}H_{12}O_3$	**-**	227	143	185	19.86	0.039-2
quadrangularin B (**2**)	$C_{30}H_{28}O_7$	**-**	499	359	255, 289	19.57	0.0048-1.25
δ-viniferin (**3**)	$C_{28}H_{22}O_6$	**-**	453	361	227, 107, 215	21.57	0.0097-1.25
oxistilbenin F (**4**)	$C_{30}H_{28}O_7$	**-**	499	227	271, 453, 225	22.14	0.0048-1.25
oxistilbenin G (**5**)	$C_{30}H_{28}O_7$	**-**	499	453	227, 271, 225	22.41	0.0048-1.25

Table 4. Stilbene levels in wine before and after heat treatment (24h at 30°C). Results are expressed in µg/L as means of 3 instrumental replicates ± SD.

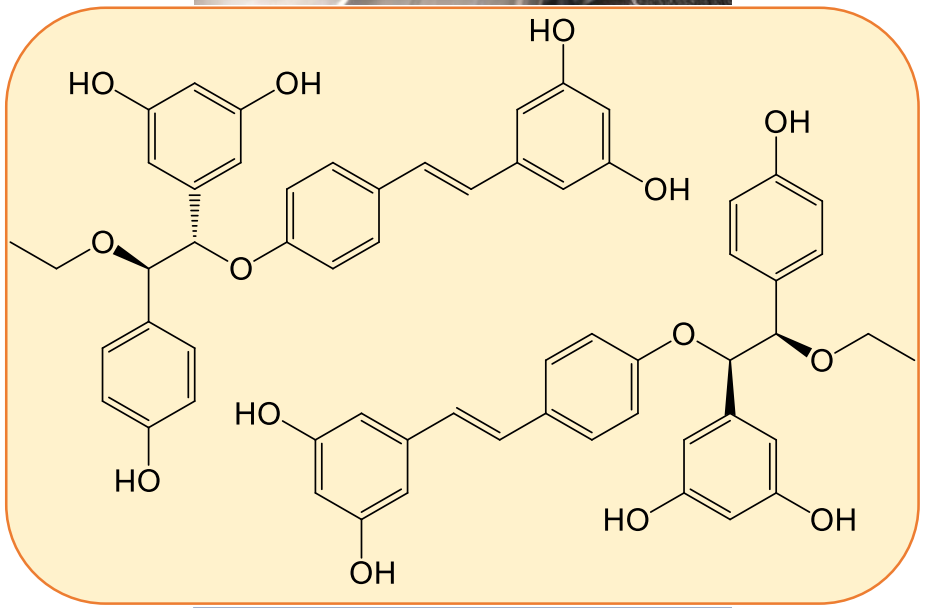
Compound	**Before heating**	**After heating**
resveratrol (**1**)	330 ± 100	160 ± 100
quadrangularin B (**2**)	nd	nd
δ-viniferin (**3**)	20 ± 10	100 ± 10
oxistilbenin F (**4**)	nd	nd
oxistilbenin G (**5**)	nd	6 ± 1
Total	350 ± 110	266 ± 111



Resveratrol oxidative coupling

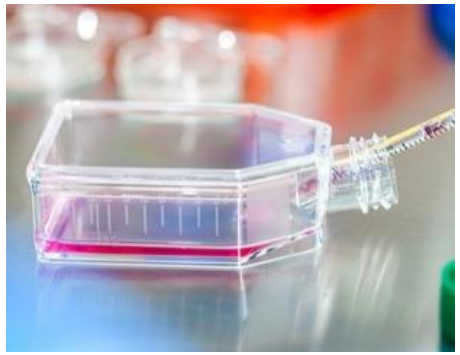
Metals

Ethanol



Identification of new resveratrol dimers

Investigation of their presence in red wine



Study of their anti-inflammatory activity *in vitro*