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Abstract

The present work aimed at optimizing a two-step enzymatic plus solvent-based process for the recovery of bioactive compounds from white grape (*Vitis vinifera* L., mix of Trebbiano and Verdicchio cultivars) pomace, the winemaking primary by-product. Phenolic compounds solubilised by water enzyme-assisted and ethanol-based extractions of wet (WP) and dried (DP) pomace were characterized for composition and tested for antioxidant, anti-tyrosinase and anti-inflammatory bioactivities. Ethanol treatment led to higher phenol yields than water extraction, while DP samples showed the highest capacity of releasing polyphenols, most probably as a positive consequence of the pomace drying process. Different compositions and bioactivities were observed between water and ethanol extracts and among different treatments and for the first time the anti-tyrosinase activity of *V. vinifera* pomace extracts, was here reported. Enzymatic treatments did not significantly improve the total amount of solubilised compounds; Celluclast in DP led to the recovery of extracts enriched in specific compounds, when compared to control. The best extracts (enzymatic plus ethanol treatment total levels) were obtained from DP showing significantly higher amounts of polyphenols, flavonoids, flavanols and tannins and exerted higher antioxidant and anti-tyrosinase activities than WP total extracts. Conversely, anti-inflammatory capacity was only detected in water (with and without enzyme) extracts, with WP samples showing on average a higher activity than DP. The present findings demonstrate that white grape pomace constitute a sustainable source for the extraction of phytochemicals that might be exploited as functional ingredients in the food, nutraceutical, pharmaceutical or cosmetic industries.

Keywords: Antioxidant activity; anti-tyrosinase activity; by-product; enzymatic digestion; white grape pomace; phenols.

Abbreviations: AA: ascorbic acid; Abs: absorbance; ABTS: 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; CAT: catechin; DP: dried pomace; DW: dry weight; GA: gallic acid; KA: kojic acid; WP: wet pomace.

1. Introduction

The large volumes of both liquid and solid by-products generated by the food processing industry poses major environmental and economic challenges. The waste load at a food processing plant can be significantly reduced through the application of new or modified industrial processes and reuse methods especially developed to convert by-products into added-value biomolecules and biofuels [1]. In fact, food by-products contain numerous high-value compounds, which could be reused in the food, feed, cosmetic and pharmaceutical industries. Grape (*Vitis* sp.) pomace constitutes the major by-product of the wine making process, representing generally 20-30% of the processed grapes weight [1]. It is mainly made up of pressed skins, seeds and stems of the fruit with a composition that varies considerably depending on grape variety and winemaking technology. Winemakers sometimes produce spirit from grape by-products, but pomace is also traditionally used as animal feed or fertilizer [1, 2]. It has been estimated that more than 9 million tons of grape pomace are generated annually [3], which causes a serious environmental and disposal problem for wineries. However, solid by-products from wine industry are rich in phenolic compounds, in particular flavonoids (such as anthocyanins and flavanols), phenolic acids and stilbenes [4-6], many of which have been shown to be beneficial to human health due to their antioxidant capacity which is believed to contribute to the prevention of cancer and cardiovascular diseases [7]. Anti-inflammatory, anti-microbial and anti-cholesterol properties have also been attributed to grape polyphenols [4, 5, 8]. Due to these properties, there is a vast array of potential applications for grape pomace extractable components: as ingredients of functional foods and feeds, cosmetics and nutraceuticals; and as natural colorants and preservatives of foods [4, 6]. Moreover, nowadays there is a growing interest in finding phytochemicals than can be used as an alternative to synthetic substances, which are sometimes perceived by consumers as harmful to human health [1, 2].

Solvent (mainly water, ethanol and methanol) and supercritical fluid extractions are the most efficient in recovering phenolic compounds from grape pomace [2, 9, 10]. Available studies regarding phenolic compositions, extractions and applications are mainly focused on pomace from red grape

varieties, whereas little attention has been devoted to white grape pomace, which also contains a wide spectrum of potentially bioactive phenols. Moreover, to the authors' knowledge, phenolic extraction by means of cell wall polysaccharide degrading enzyme mixtures has only been reported for red pomace [3, 5, 11]. In contrast to red wines, white wines are usually made by immediately removing, after grape pressing, the pomace from the must. As a consequence, a much lower phenolic content is present in white wines with respect to red ones. The differences between red and white wine production processes may also affect the amount and availability of polyphenols in the resulting pomace. In fact, as white grape must is not usually fermented in contact with the solid parts of the grape, higher contents of phenolic compounds are present in white compared to red pomace [12, 13].

In the present work, enzyme-assisted and ethanol-based extractions were combined in a two-step process aimed at the recovery of phenolic compounds from wet pomace (WP) and dried pomace (DP) of white grapes (*Vitis vinifera* L., mixture of Trebbiano and Verdicchio cultivars). The extracts with the highest amount of compounds were characterised for phenolic composition and tested for antioxidant, anti-tyrosinase and anti-inflammatory bioactivities.

2. Materials and Methods

2.1. Materials

White pomace, derived from a mix (60:40) of *V. vinifera* cv. Trebbiano and Verdicchio, was supplied by the Cantine Moncaro wineries (Jesi, Ancona, Italy) immediately after wine production. Pomace was either frozen (wet pomace, WP) or dried (dried pomace, DP) in an industrial vented oven (60°C for 24h) and stored at -20°C until used for analyses. Different WP and DP lots were pulled together before grinding in order to minimise the biological differences.

2.2. Enzyme-assisted and ethanol-based pomace extractions

White pomace was treated as previously described by Ferri et al. [5]. WP was ground in a kitchen blender with the addition of distilled water (1:5 g/mL), while DP was ground directly and rehydrated with distilled water (1:5 g/mL for 1h) just before enzymatic digestion. Enzyme-assisted extractions of WP and DP pomace suspensions (20 mL aliquots) were carried out by adding different concentrations (0.5, 1 or 2% enzyme volume/pomace DW) of Pectinex 3XL, Pectinex Ultra SPL, Termamyl, Fungamyl, Pentopan 500BG or Celluclast (Sigma-Aldrich, Milan, Italy). The enzymatic treatments were incubated on an orbital shaker (150 rpm) at different incubation times (2, 6, 24h) and at each enzyme's optimal working temperature (24, 30 or 37°C) [5]. Controls without the addition of enzymes, were also prepared. After incubation, the supernatant (water extract) and pellet fractions were collected [5]. The pellet was extracted overnight with 30 mL of 95% v/v ethanol at 24°C, the supernatant was separated from the pellet and stored at -20°C until further analyses.

2.3. Extract characterisation

Water and ethanol extracts were characterised spectrophotometrically for phenolic [14, 15], flavonoid [15, 16] and flavanol [17] contents. The results were expressed, respectively, as gallic acid (GA) and catechin (CAT) equivalents by means of calibration curves. The amount of tannins was analysed according to Porter et al. [18]; briefly, two aliquots of 0.3 mL of each sample were mixed with 0.9 mL of reagent (50% *n*-butanol, 50% 12N HCl, 0.015% $FeCl_3$) and incubated for 30 min, one at room temperature and the second at 100°C. Absorbance (Abs) was measured at 550 nm and tannin content (g/L) was calculated as ($Abs_{100°C} - Abs_{room\ temperature}$) x 0.1736.

Both water and ethanol supernatants were analysed for phenolic compounds by HPLC-DAD as previously described [5, 19]. The adopted HPLC-DAD separation procedure allowed for the simultaneous analysis of 28 different compounds among stilbenes, phenolic acids and flavonoids [19]: *trans*- and *cis*-resveratrol, *trans*- and *cis*-piceid, *trans*- and *cis*-resveratroloside, piceatannol; gallic, protocatechuic, syringic and vanillic acids; caffeic, chlorogenic, *p*-coumaric, ferulic, sinapic and *trans*-

cinnamic acids; catechin, epicatechin, epigallocatechin gallate (EGCG), epicatechin gallate, epigallocatechin; vanillin, naringenin, quercetin, rutin, myricetin and kaempferol.

2.4. Determination of biological activities

In vitro antioxidant activities were measured using the 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method with minor modifications [15]. The results were expressed as ascorbic acid (AA) equivalents by means of a dose-response calibration curve.

Anti-tyrosinase activity was assessed by an optimised tyrosinase enzyme inhibition assay [20]. The kinetics of brown colour formation was evaluated by absorbance measurement (490 nm) in reactions containing 10 U of tyrosinase and 2 mM L-DOPA (the substrate) in the presence of the sample. The results were expressed as kojic acid (KA, a well-known tyrosinase inhibitor) equivalents by means of a calibration curve (between 1 and 10 μg of KA).

A bioluminescent cell-based assay for anti-inflammatory activity was performed using human embryonic kidney HEK293 cells ATCC (American Type Culture Collection, Manassas, VA, USA) routinely grown in Dulbecco modified essential medium (DMEM high glucose 4.5 g/L, GE Healthcare) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/μL penicillin, and 50 μg/mL streptomycin. HEK293 cells were plated in 96-well plates the day before transfection at a density of $2x10^4$ cells/100 μL of growth medium per well. Co-transfection was performed with plasmid pGL4.32[luc2P/NF-κB-RE/Hygro] containing five copies of the NF-κB response element (NF-κB-RE) driving transcription of the luc2P reporter protein (Promega, Madison, WI) and with plasmid pcDNA.3.1-mcherryPRET9 expressing a thermostable mutant *P. pyralis* luciferase [21], and by using FuGENE®HD (Promega) according to the manufacturer's instructions. During co-transfection, cells were incubated for 24h at 37°C with 5% CO_2. After transfection cells were co-incubated for 5h with 100 μL of fresh medium containing sample (1:5 and 1:20 dilutions) and 1 ng/mL TNFα (Sigma Aldrich, Saint Louis, Missouri, USA). Bioluminescent (BL) measurements were performed with a Varioskan™ Flash Multimode Reader acquiring BL signals with band pass green

and red filters after injection of 100 µL substrate BrightGlo (Promega). Corrected values were obtained as previously described [5].

2.5. Statistical analyses

All the treatments and assay procedures were performed twice with two biological replicates each. Each repetition was analysed in two technical replicates. The results are expressed as the mean (n=2) ± SD. Cell-based assays were performed in triplicate, with at least three technical replicates ((n=3) ± SD). Statistically significant differences between data sets were analysed by using one-way ANOVA test followed by post-hoc corrected two tail *t*-student test assuming equal variance.

3. Results

3.1. Enzyme-assisted and ethanol white pomace extractions

Different concentrations (0.5, 1 or 2% v/w) of six commercial enzymes (Pectinex 3XL, Pectinex Ultra SPL, Termamyl, Fungamyl, Pentopan, Celluclast) were tested in enzyme-assisted extractions of both wet pomace (WP) and dried white pomace (DP) in 2h incubations, followed by ethanol extraction. Subsequently, the Folin-Ciocalteu assay was used to quantify phenolic compound content of both aqueous and ethanol extracts (Suppl. Fig. 1). As expected, 95% (v/v) ethanol was able to improve the extraction efficiency of phenolic compounds from the residual pellet with respect to pure water-based (with or without enzyme) treatments (Suppl. Fig. 1). In WP samples, the phenolic content was on average 2.2-fold higher in ethanol compared to water extracts (Suppl. Fig. 1A), while only a 1.7-fold average increase was obtained in ethanol extracts of DP samples (Suppl. Fig. 1B). In general, DP contained 2-fold more extractable phenols than WP. Larger variations were observed among WP samples, with total amounts (water plus ethanol extracts) ranging between 940 and 1494 mg GA eq/L (Suppl. Fig. 1A), while DP total phenols were between 1995 and 2437 mg GA eq/L (Suppl. Fig. 1B). Overall, the best treatments (enzymatic plus ethanol extracts total levels) for phenolic compounds were

2% Pectinex 3XL 24°C, 2% Pentopan 30°C and 2% Celluclast 37°C (Suppl. Fig. 1). To increase extraction yields, the best enzymatic treatments and related controls for WP and DP were performed at 2, 6 and 24h incubation times, and the total level of phenolic compounds in water supernatant and ethanol extracts, were measured (Suppl. Fig. 2). For WP the highest total phenolic contents were detected in samples incubated at 2h or 6h (on average 1316 and 1272 mg GA eq/L, respectively), while a 25% decrease was found in 24h treated samples (Suppl. Fig. 2A). On the other hand, in DP a no statistically significant difference was detected among samples at incubated 2, 6 and 24h (on average 2635, 2659 and 2412 mg GA eq/L, respectively) (Suppl. Fig. 2B).

3.2. Total phenolic contents

On the basis of phenol yield, the following best conditions (two-step enzymatic plus ethanol treatments) for both WP and DP, were selected (Suppl. Fig. 2): for WP 2h 2% Pectinex 3XL 24°C, 2h control 30°C, 6h control 30°C, 6h 2% Pentopan 30°C and 2h control 37°C for WP; for DP 2h control 37°C, 2h 2% Celluclast 37°C and 6h 2% Celluclast 37°C.

Both water and ethanol extracts were characterised by spectrophotometric assays (Fig. 1). On average, the phenolic content was 1.5-fold higher in ethanol with respect to water extracts and 2.1-fold higher in DP than in WP samples (Fig. 1A). Similar trends were obtained for flavonoids (Fig. 1B), flavanols (Fig. 1C) and tannins (Fig. 1D) contents. Flavanols (such as flavan-3-ols or catechins), a subclass of flavonoids, constituted the majority of flavonoids extracted from Trebbiano and Verdicchio pomace, especially in DP (Fig. 1B-C), confirming that they represent the most abundant phenolic group present in white grape pomace [22]. Moreover, the level of flavanols seemed to decrease with increasing incubation time and/or temperature and following enzyme digestion (Fig. 1C). This can probably be explained considering the fact that the structure of phytochemicals, such as phenolic acid and flavonoids present in fruits, vegetables and grains both in the free and bound forms, is degraded or modified during thermal and non-thermal processing [23]. The maximum level of solubilised total (water plus ethanol extracts) tannins was detected for DP in 2h control 37°C (2120 mg/L) and for WP

in 2h control 30°C (1477 mg/L) (Fig. 1D), with a general pattern similar to that observed for flavanols (tannin monomers) (Fig. 1C-D).

3.3. Characterization of individual phenolic compounds by HPLC-DAD

Water and ethanol extracts of the eight best treatments (Fig. 1) were also analysed by HPLC-DAD to quantify specific phenolic compounds (Table 1). In WP samples, the detected phenols were more abundant in ethanol than in water extracts (up to a maximum 36.2-fold difference for *cis*-resveratroloside), with the only exception of gallic acid. In WP, the incubation time increase negatively affected the metabolite extraction yield, while no effect was detected in relation to temperature. In fact, WP 2h control 30°C and WP 2h control 37°C results were not statistically significantly different, while most of the detected compounds in WP decreased after 6h at 30°C when compared to 2h control 30°C. In DP, some phenolic compounds (in particular those belonging to the catechin family) were extracted at higher concentrations in water samples as a consequence of the enzymatic digestions (2h or 6h 2% Celluclast 37°C) when compared to 2h Control 37°C (Table 1). Both water and ethanol solvents were generally able to extract phenolics more efficiently from DP than from WP, such as the up to 110-fold higher amount of catechin in DP water samples compared to the corresponding WP samples. Moreover, a wider spectrum of compounds was detected in DP extracts compared to WP (Table 1). In all the analysed samples, a large amount of the unidentified chromatographic peaks showed an absorbance spectrum characteristic of flavanols [24], thus confirming that these compounds are the main phenolic group present in white grape pomace, as also observed by spectrophotometric analyses (Fig. 1).

While most of the detected phenols (Table 1) have been previously found in white pomace extracts from other grape cultivars [11-13, 22, 25], the presence of mono-glycoside stilbenes (*cis*-piceid and *cis*-resveratroloside) is here reported for the first time.

3.3. Biological activities of the extracts

In agreement with spectrophotometrical analyses (Fig. 1), WP samples showed on average a 1.7-fold lower total (water plus ethanol extracts) antioxidant activity than DP samples, while ethanol extracts of both WP and DP showed a higher capacity than water extracts (Fig. 2A). The highest antioxidant activity was detected in the DP 6h 2% Celluclast 37°C sample (water plus ethanol extracts total activity of 7.82 g AA eq/L) (Fig. 2A).

The anti-tyrosinase activity of *V. vinifera* pomace extracts was also determined (Fig. 2B). In agreement with biochemical (Fig. 1) and antioxidant capacity results (Fig. 2A), ethanol extracts were more active than water ones (up to 5.3-times in WP 2h 2% Pectinex 24°C), and DP samples were more active than WP ones (on average 2.8-times; 686.3 and 243.3 mg KA eq/L, respectively) (Fig. 2B). The percentage of tyrosinase enzyme inhibition ranged from 43 to 71% in water and from 63 to 79% in ethanol samples.

Anti-inflammatory activity of both water and ethanol extracts (1:20 and 1:5 dilutions) was investigated using a cell-based assay. Significant bioactivity was identified for most of the water extracts at the 1:5 dilution (Fig. 3), where a decrease in reporting gene transcription was observed in treated cells in comparison with control. Water WP samples showed on average a higher anti-inflammatory activity than DP. In particular, WP 2h control 37°C was able to decrease TNFα-induced inflammation by 62% (Fig. 3). No activity was reported for ethanol extracts at both dilutions and for water extracts at the 1:20 dilution that showed no variation in transcription levels respect to controls.

4. Discussion

In the present paper, the recovery of bioactive phenolic compounds from wet (WP) and dried (DP) white grape (*Vitis vinifera* L.) pomace was investigated by means of different optimised processes including a first extraction step in water (with or without enzymes) followed by a second one in ethanol.

DP samples showed the highest capacity of releasing plant compounds, which included high amounts of phenols, flavonoids, flavanols and tannins (Fig. 1). The biochemical compositions of

extracts depended on the kind of solvent (water and ethanol) and treatment (with or without enzymes) used (Fig.1 and Table 1).

Previous published data showed that the feedstock preserving method had a significant impact on the recovery of phenolic compounds from berry pomace. In particular, drying of red grape pomace caused a significant reduction in the amount of extractable molecules, indicating that moisture content is important to facilitate solvent diffusion and reaching a kinetic equilibrium [5, 10]. In contrast to what has been reported for red pomace [5, 10], the present data (Fig. 1) indicate that, at least for Trebbiano and Verdicchio pomace, drying could be a useful technique to preserve white grape pomace until further industrial application, with the positive consequence of increasing phenol yields after extraction. However, the reduction of moisture content after dehydration, the severity of heat application and the rate of evaporation may influence the stability of water-soluble phenolic compounds, which can result in opposite effects (increase or decrease of recovery) in different samples [23]. These considerations, together with the different metabolite composition of red and white grape winery residues, may explain the different results obtained, after similar extraction treatments, between white (Fig. 1) and red pomace [5].

To the authors' knowledge, the present work represents the first study on enzyme-assisted extraction of phenols from white grape pomace. Results indicated that the enzyme treatments did not significantly improve the total amount of solubilised compounds, in agreement to previous results on red grape pomace [5]. Nevertheless, some specific enzyme-treatments produced extracts enriched in specific compounds, such as 2h and 6h 2% Celluclast to DP, and 6h 2% Pentopan to WP (Table 1). In particular, Celluclast is a commercial mixture containing several enzymes (such as ferulic acid esterase and glycosidase) that may selectively release or modify the structure of certain polyphenols thereby also promoting their antioxidant activity [3]. Previous research using cell wall degrading enzymes to solubilise phytochemicals from red grape pomac reported different results depending on plant sample (e.g. cultivar, agricultural and oenological practices) and on adopted extraction process [2, 3, 5, 11]. For Sangiovese and Montepulciano [5] and for Merlot red WP [3], increasing incubation times

negatively affected metabolite extraction probably due to metabolite degradation during extended incubation. In particular, increasing incubation times (8 to 48h) led to a loss of up to 27% of phenols extracted from Merlot pomace after treatment with or without Celluclast [3]. Present data report a decrease in flavonoids, flavanols and tannins recovery (Fig. 1B-D) during extended incubations, therefore, confirming previous findings.

Overall, a comparison between the present results (Fig. 1) and data on red grape pomace obtained by similar methodologies [5], indicate that white grape pomace extracts possess higher levels of phenols (1.4 and 7.7-fold, respectively, for WP and DP; red WP total phenols were on average 990 mg GA eq/L and red DP ones were 393 mg GA eq/L), flavonoids (1.4 and 5.1-fold, respectively, for WP and DP; red WP total flavonoids were on average 882 mg CAT eq/L and red DP ones were 420 mg CAT eq/L) and tannins (2.1 and 5.4-times, respectively, for WP and DP; red WP total tannins were on average 577 mg/L and red DP ones were 350 mg/L) with respect to red grape pomace samples. The present findings are agreement with previous studies performed on four different Chilean varieties and on Vidal Blanc and Viognier cultivars, in which higher polyphenols [12, 13], flavonoids [13] and total proanthocyanidins [12] concentrations, were also detected in white with respect to red grape pomace. Besides the obvious differences related to the used grape cultivars, these results may also be ascribed to a more efficient phenol extraction during the red wine making process in which the fermentation is performed in the presence of the pomace. In contrast, white wine fermentation is generally performed in the absence of berry skins and seeds, thus resulting in a higher content of phenols still present in the pomace.

All the extracts exerted antioxidant and anti-tyrosinase activities (Fig. 2). On average, WP samples showed a 1.7-fold lower total antioxidant activity (water plus ethanol extracts) than DP samples. This result could be explained by the fact that high temperature drying treatments have significant effects on the stability and composition of naturally occurring antioxidants [23]. The antioxidant capacity of different white grape pomace extracts was previously measured by using several *in vitro* methods [2]. In particular, using the ABTS method, antioxidant activities were reported for aqueous/ethanol pomace

extracts of four different white grape varieties from the Balearic Islands (72-134 mg Trolox/gDW equivalent to 0.03-0.05 g Trolox/L) [25], methanol extracts of Palomino Fino and Moscatel marcs (20 mM Trolox equivalent corresponding to 5 g Trolox/L) [26], and 80% v/v acetone samples from Vidal Blanc and Viognier cultivars (334-951 μmol Trolox/gDW equivalent to 21-60 g Trolox/L) [13]. In all these studies ABTS scavenging capacity was positively correlated with phenol, flavonoid and condensed tannin contents. A comparison between the antioxidant activity of white (Fig. 2A) and red pomace [5] extracted by the same process, demonstrated on average a 1.6 and 4.9-times higher level respectively, for WP and DP white samples (antioxidant capacities were on average 2.7 and 1.5 g AA eq/L in red WP and DP extracts respectively). This finding is also in agreement with recent investigations of de la Cerda-Carrasco [12], in which the strong oxygen radical scavenging capacity of a white pomace extract (Chardonnay, 80% v/v ethanol extract) and its flavonoid and phenolic contents was positively correlated to the selective *in vivo* inhibition of intestinal α-glucosidases and to the suppression of the postprandial hyperglycemia in diabetic mice, thus also suggesting the potential medical applications of pomace extracts [27].

For the first time, the anti-tyrosinase activity of *V. vinifera* pomace extracts is here reported. Tyrosinase is a multifunctional oxidase which catalyses the first two steps of mammalian melanogenesis and is responsible for the enzymatic browning reactions in damaged fruits during post-harvest handling and processing [28]. Both phenomena (hyperpigmentation in human skin and browning in fruits) are considered not desirable and, therefore, new potent tyrosinase inhibitors are actually being sought. One important endpoint for bioactive phytochemical ingredients is in fact their ability to decrease skin pigmentation and/or to inhibit fruit browning during post-harvest processing. This feature has been related to many different phenolic compounds. Most of the identified compounds in the present extracts (Table 1) are known tyrosinase inhibitors [25-27], such as quercetin and gallic acid, having the highest inhibitory activity, followed by resveratrol and catechin derivatives and protocatechuic acid with the lowest inhibitory effect. However, depending on their structure, polyphenols may act both as inhibitors or substrates of tyrosinase [28]. Regarding grape extracts, anti-

tyrosinse capacity was previously measured in aqueous/ethanol extracts from pomace of two cultivars of *Vitis rotundifolia* [29] and in *V. vinifera* seed and peel extracts [30]. In particular, the latter showed an inhibition of tyrosinase ranging from 15 to 35%, about half of that measured in Verdicchio and Trebbiano pomace extracts (between 43 and 71% inhibition in water extracts and from 63 to 79% in ethanol samples).

Anti-inflammatory capacity was only detected in water extracts (Fig. 3), probably as a consequence of a different phenolic composition of the samples. These data are consistent with previous studies on animal models demonstrating that white and red grape pomace extracts were able to suppress, in a dose-dependent manner, chronic inflammation induced by lipopolysaccharide (LPS) and galactosamine (GalN) in N13 microglia cells [8] and in Sprague-Dawley rats [31]. Although more investigations are required to evaluate if the reported anti-inflammatory activity is concentration-dependent, the present results seem promising and lead us to hypothesise a possible use of pomace extracts as ingredients or supplements for functional foods. Future sample characterization will enable to investigate if the single components, such as quercetin, catechin, epicatechin and gallic acid, are responsible for such activity.

In conclusion, the present results strongly support a possible application of white grape pomace extracts, obtained by a two-step process, as an inexpensive, easily available and alternative source of bioactive phytochemicals, which could be advantageously used as ingredients in the food, nutraceutical, pharmacological or cosmetic fields.

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Supplementary material captions

Supplementary Figure 1. Levels of phenolic compounds in water supernatants and ethanol extracts of (A) wet pomace (WP) and (B) dried pomace (DP) samples treated for 2h with different concentrations (0.5, 1 or 2% enzyme volume/pomace DW) of six commercial enzymes and controls. The data are expressed as mg of gallic acid (GA) equivalent per litre of extract (mg GA eq/L). The star symbol indicates a statistically significant difference (one way ANOVA followed by post hoc two tail Student's *t*-test, $p<0.05$) between the total level (water plus ethanol extracts) of each enzymatic treated sample with respect to the control incubated at the same temperature (24, 30 or 37°C). Data are the mean ± SD (n=2).

Supplementary Figure 2. Levels of phenolic compounds in water supernatants and ethanol extracts of the three best treatments and the three controls of (A) wet pomace (WP) and (B) dried pomace (DP) samples incubated for increasing times (2, 6 and 24h). The data are expressed as mg of gallic acid (GA) equivalent per litre of extract (mg GA eq/L). The star symbol indicates a statistically significant difference (one way ANOVA followed by post hoc two tail Student's *t*-test, $p<0.05$) between the total level (water plus ethanol extracts) of the treatments incubated for 6h and 24h in comparison to the same treatment at 2h. Data are the mean ± SD (n=2).

Figure Captions

Figure 1. Levels of total (water plus ethanol extracts) (A) phenols, (B) flavonoids, (C) flavanols and (D) tannins in water and ethanol extracts of the five best wet pomace (WP) and the three best dried pomace (DP) samples. Phenol data are expressed as mg of gallic acid (GA) equivalent per L of extract (mg GA eq/L); flavonoid and flavanol data are expressed as mg of catechin (CAT) equivalent per L of extract (mg CAT eq/L); tannins are expressed as mg of tannin per L. Different letters indicate statistically significant difference (one way ANOVA followed by post hoc two tail Student's *t*-test, $p<0.05$) between data total levels (water plus ethanol extracts). Data are the mean ± SD (n=2).

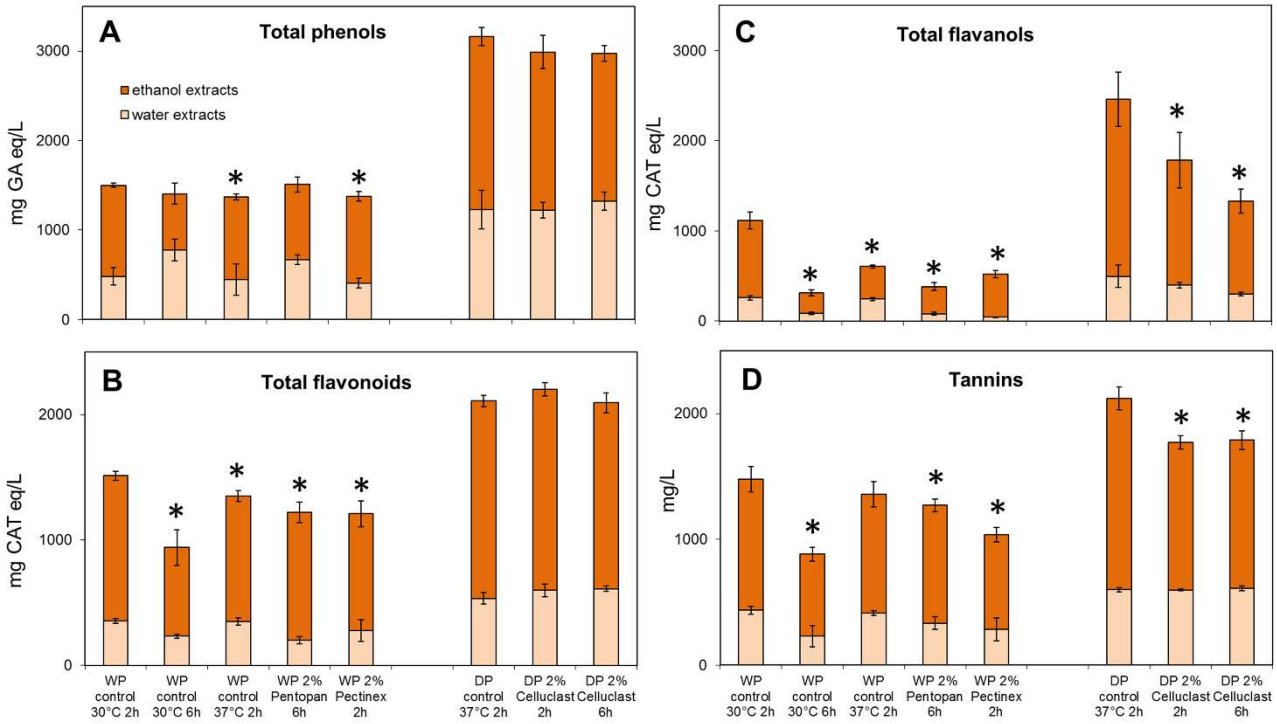
Figure 2. (A) Antioxidant and (B) anti-tyrosinase activities in selected wet pomace (WP) and dried pomace (DP) extracts. Antioxidant data were obtained by means of the ABTS method and are expressed as g of ascorbic acid (AA) equivalent per L of extract (g AA eq/L). Anti-tyrosinase data are expressed as mg of kojic acid (KA) equivalent per L of extract (mg KA eq/L). Different letters indicate statistically significant difference (one way ANOVA followed by post hoc two tail Student's *t*-test, $p<0.05$) between data total levels (water plus ethanol extracts). Data are the mean ± SD (n=2).

Figure 3. Anti-inflammatory activity obtained with HEK293 cells co-transfected with pGL4.32[luc2P/NF-κB-RE/Hygro] and pcDNA.3.1-mcherryPRET9 and co-incubated for 5h at 37°C with 1:5 WP and DP water sample dilutions in the presence of 1 ng/mL TNFα. Control sample (H_2O Control) for inflammatory activity was obtained by incubating cells with 1 ng/mL TNFα and the BL signal of the control well was set at 1 to normalize results and enable both evaluation of anti- and pro-inflammatory activities. Different letters indicate statistically significant difference (one way ANOVA followed by post hoc two tail Student's *t*-test, $p<0.05$) between data total levels (water plus ethanol extracts) and the H_2O Control. Data are the mean ± SD (n=3).

Table 1. Polyphenol levels (mg/L) of water and ethanol extracts of wet (WP) and dried (DP) white grape pomace determined by HPLC-DAD analysis. Different letters indicate statistically significant difference (one way ANOVA followed by post hoc two tail Student's *t*-test, $p<0.05$) between data. Data are the mean ± SD (n=2).

Compound	Type of extract	WP 2h 2% Pectinex 3XL 24°C	WP 2h Control 30°C	WP 6h Control 30°C	WP 6h 2% Pentopan 30°C	WP 2h Control 37°C	DP 2h Control 37°C	DP 2h 2% Celluclast 37°C	DP 6h 2% Celluclast 37°C
Catechin	Water	0.26 ± 0.02 ^{a}	0.65 ± 0.02 ^{b}	0.34 ± 0.03 ^{a}	0.48 ± 0.06 ^{c}	0.67 ± 0.01 ^{b}	45.66 ± 2.01 ^{d}	55.43 ± 5.42 ^{d,e}	57.61 ± 1.29 ^{e}
	Ethanol	3.39 ± 0.22 ^{a}	5.76 ± 1.79 ^{b}	3.38 ± 0.14 ^{a}	3.20 ± 0.24 ^{a}	5.18 ± 0.93 ^{b}	31.62 ±0.94 ^{c}	32.29 ± 3.50 ^{c}	31.64 ± 2.25 ^{c}
Epicatechin	Water	0.62 ± 0.04 ^{a}	0.86 ± 0.04 ^{b}	0.70 ± 0.06 ^{a}	0.86 ± 0.05 ^{b}	0.83 ± 0.15 ^{b}	31.86 ±0.17 ^{c}	41.39 ± 3.92 ^{d}	42.90 ± 1.30 ^{d}
	Ethanol	2.97 ± 0.22 ^{a}	4.64 ± 0.70 ^{b}	2.69 ± 0.20 ^{a}	3.04 ± 0.01 ^{a}	4.28 ± 0.75 ^{b}	26.96 ± 0.03 ^{c}	27.47 ± 1.39 ^{c}	27.26 ± 0.34 ^{c}
Epigallocatechin gallate	Water	1.32 ± 0.51 ^{a}	2.99 ± 0.08 ^{b}	1.47 ± 0.12 ^{a}	2.03 ± 0.08 ^{a}	2.28 ± 1.32 ^{a,b}	19.27 ± 1.27 ^{c}	45.06 ± 18.60 ^{d}	48.69 ± 5.58 ^{d}
	Ethanol	8.31 ± 0.72 ^{a}	13.58 ± 2.86 ^{b}	8.15 ± 2.21 ^{a,b}	9.62 ± 0.67 ^{a}	10.62 ± 1.44 ^{a,b}	35.42 ± 0.47 ^{c}	36.23 ± 3.16 ^{c}	37.39 ± 1.80 ^{c}
Epicatechin gallate	Water	0.45 ± 0.03 ^{a}	0.65 ± 0.02 ^{b}	0.49 ± 0.04 ^{a}	0.57 ± 0.05 ^{b}	0.68 ± 0.11 ^{b}	2.12 ± 0.76 ^{c}	2.81 ± 0.22 ^{c}	3.03 ± 0.14 ^{c}
	Ethanol	7.15 ± 0.12 ^{a}	10.00 ± 1.17 ^{a,b}	8.05 ± 1.64 ^{a}	10.45 ± 0.11 ^{b}	10.50 ± 0.20 ^{b}	13.97 ± 0.13 ^{c}	13.33 ± 0.75 ^{c}	12.90 ± 0.36 ^{c}
Epigallocatechin	Water	0.22 ± 0.01 ^{a}	0.31 ± 0.01 ^{b}	0.26 ± 0.08 ^{a,b}	0.30 ± 0.02 ^{b}	0.32 ± 0.01 ^{b}	4.82 ± 0.16 ^{c}	5.44 ± 3.36 ^{c,d}	7.82 ± 0.56 ^{d}
	Ethanol	7.94 ± 0.05 ^{a}	9.66 ± 3.75 ^{a}	8.22 ± 1.70 ^{a}	10.64 ± 0.81 ^{a,b}	10.74 ± 0.09 ^{b}	35.74 ± 0.18 ^{c}	39.33 ± 2.53 ^{c}	36.37 ± 0.05 ^{c}
Gallic acid	Water	0.20 ± 0.01 ^{a}	0.45 ± 0.05 ^{b,c}	0.25 ± 0.01 ^{a}	0.37 ± 0.03 ^{b}	0.56 ± 0.06 ^{c}	22.73 ± 0.66 ^{d}	23.70 ± 0.63 ^{d}	26.62 ± 0.76 ^{d}
	Ethanol	-	-	-	-	-	-	-	-
Protocatechuic acid	Water	-	-	-	-	-	1.89 ± 0.09 ^{a}	2.74 ± 0.54 ^{b}	2.41 ± 0.02 ^{b}
	Ethanol	-	-	-	-	-	1.01 ± 0.13 ^{a}	1.48 ± 0.03 ^{b}	1.50 ± 0.18 ^{b}
Chlogenic acid	Water	-	-	-	-	-	9.21 ± 0.61 ^{a}	10.85 ± 1.38 ^{a,b}	12.57 ± 1.08 ^{b}
	Ethanol	-	-	-	-	-	-	-	-
***Cis*-Piceid**	Water	0.33 ± 0.01 ^{a}	0.48 ± 0.03 ^{b}	0.35 ± 0.01 ^{a}	0.42 ± 0.01 ^{b}	0.48 ± 0.07 ^{b}	1.81 ± 0.65 ^{c}	2.30 ± 0.16 ^{c}	2.42 ± 0.13 ^{c}
	Ethanol	10.56 ± 0.82 ^{a}	13.61 ± 0.18 ^{b}	9.20 ± 1.21 ^{a}	13.98 ± 0.22 ^{b}	12.54 ± 0.05 ^{b}	20.32 ± 1.16 ^{c}	23.69 ± 0.30 ^{c}	24.22 ± 0.19 ^{c}
***Cis*-Resveratroloside**	Water	0.15 ± 0.01 ^{a}	0.18 ± 0.01 ^{a}	0.16 ± 0.01 ^{a}	0.19 ± 0.01 ^{a}	0.18 ± 0.01 ^{a}	1.45 ± 0.01 ^{b}	0.74 ± 0.09 ^{c}	0.70 ± 0.21 ^{c}
	Ethanol	5.44 ± 0.30 ^{a}	6.89 ± 0.69 ^{b}	4.79 ± 0.61 ^{a}	7.38 ± 0.09 ^{b}	6.83 ± 0.51 ^{b}	11.68 ± 0.24 ^{c}	13.31 ± 0.89 ^{c}	12.21 ± 0.23 ^{c}
Quercetin	Water	-	-	-	-	-	0.46 ± 0.07 ^{a}	0.55 ± 0.05 ^{a,b}	0.67 ± 0.05 ^{b}
	Ethanol	-	-	-	-	-	-	-	-

Figure 1.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ethanol extracts
water extracts
mg GA eq/L
3000
2000
1000
0
B
Total flavonoids
mg CAT eq/L
2000
1000
0
C
Total flavanols
mg CAT eq/L
3000
2000
1000
0
D
Tannins
mg/L
2000
1000
0
WP control 30°C 2h
WP control 30°C 6h
WP control 37°C 2h
WP 2% Pentopan 6h
WP 2% Pectinex 2h
DP control 37°C 2h
DP 2% Celluclast 2h
DP 2% Celluclast 6h

Figure 2.

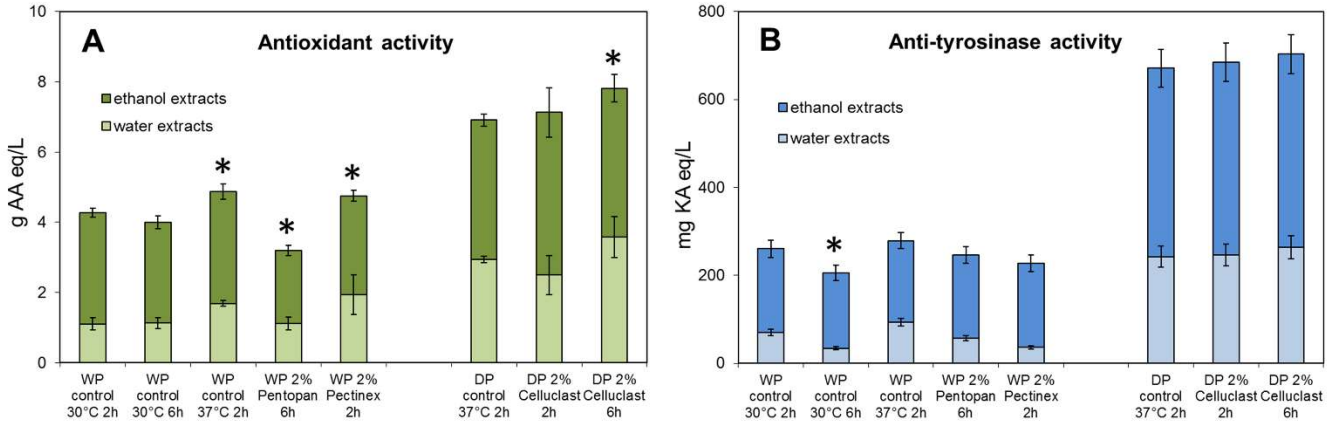
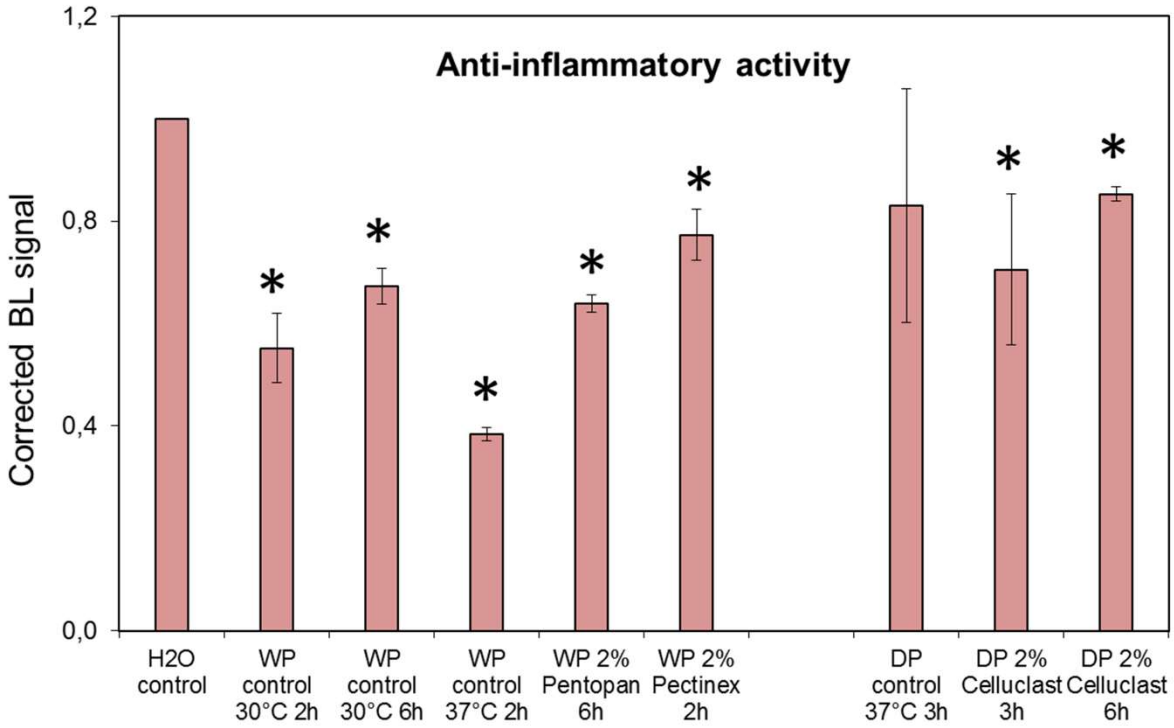
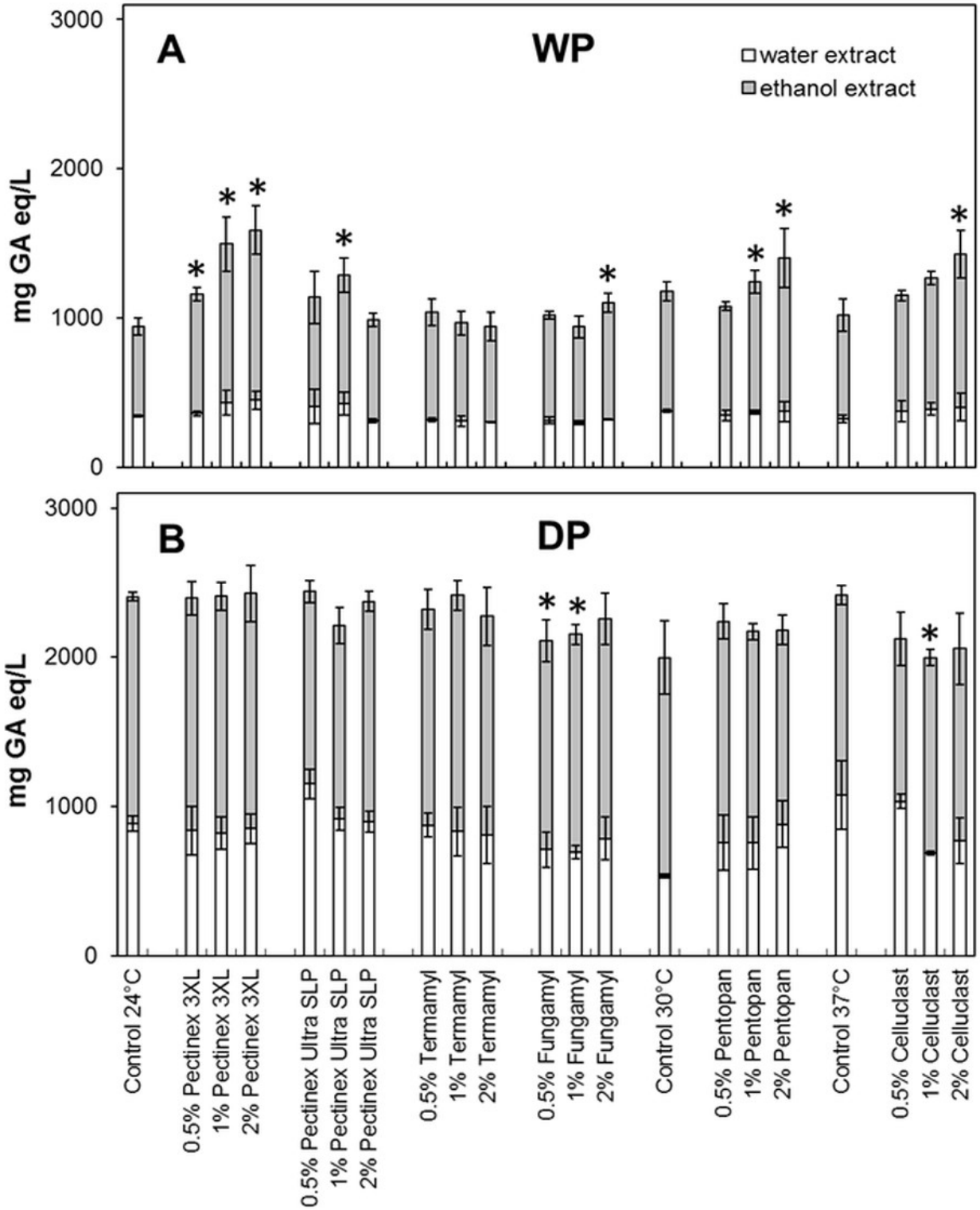

A
Antioxidant activity
ethanol extracts
water extracts
g AA eq/L
10
8
6
4
2
0
WP control 30°C 2h
WP control 30°C 6h
WP control 37°C 2h
WP 2% Pentopan 6h
WP 2% Pectinex 2h
DP control 37°C 2h
DP 2% Celluclast 2h
DP 2% Celluclast 6h
B
Anti-tyrosinase activity
ethanol extracts
water extracts
mg KA eq/L
800
600
400
200
0
WP control 30°C 2h
WP control 30°C 6h
WP control 37°C 2h
WP 2% Pentopan 6h
WP 2% Pectinex 2h
DP control 37°C 2h
DP 2% Celluclast 2h
DP 2% Celluclast 6h

Figure 3.


Anti-inflammatory activity
Corrected BL signal
1,2
0,8
0,4
0,0
H2O control
WP control 30°C 2h
WP control 30°C 6h
WP control 37°C 2h
WP 2% Pentopan 6h
WP 2% Pectinex 2h
DP control 37°C 3h
DP 2% Celluclast 3h
DP 2% Celluclast 6h

Supplementary Figure S1.



Supplementary Figure S2.