Research article

Irrigation deficit turns almond by-products into a valuable source of antimicrobial (poly)phenols

Iva Prgomet,[1,*] Berta Gonçalves,[1] Raúl Domínguez-Perles,[2] Rafaela Santos,[1] Maria José Saavedra, [1,4] Alfredo Aires,[1] Nuria Pascual-Seva,[3] Ana Barros[1]

[1] *Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro (CITAB-UTAD), 5000-801 Vila Real, Portugal; ivap@utad.pt (I.P.); bertag@utad.pt (B.G.); rafaela.santos@ciimar.up.pt (R.S.); alfredoa@utad.pt (A.A.); abarros@utad.pt (A.B.)*

[2] *Research Group on Quality, Safety and Bioactivity of Plant Foods. Department of Food Science and Technology, Centro de Edafología y Biología Aplicada del Segura – Spanish Council for Scientific Research (CEBAS-CSIC), University Campus of Espinardo Edif. 25, 30100 Murcia, Spain; rdperles@cebas.csic.es (R.D.-P.)*

[3] *Department of Plant Production, Universitat Politècnica de València, 46022 València, Spain; nupasse@prv.upv.es (N.P.-S.)*

[4] *Veterinary Sciences Department, University of Trás-os-Montes and Alto Douro (ECAV-UTAD), 5000-801 Vila Real, Portugal; saavedra@utad.pt (M.J.S.)*

*Corresponding author: email: ivap@utad.pt; Centre for the Research and Technology of Agro-Environmental and Biological Sciences, CITAB, University of Trás-os-Montes e Alto Douro, UTAD, Quinta de Prados, 5000-801 Vila Real, Portugal; Tel.: +351-259-350-800

Abbreviations: FRAP, ferric reducing ability of plasma; HPLC-DAD/UV-*Vis*, high performance liquid chromatography coupled to diode array-ultraviolet detector; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt; TPTZ, 2,4,6-tripyridyl-S′-triazine; APPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride.

ABSTRACT

Almond (*Prunus dulcis* (Mill.) D. A. Webb) production keeps an increasing trend worldwide, leading to augment in generation of harmful by-products that should be valorized as a source of bioactive phytochemicals with application in the development of new added-value products. The assessment of almond hulls and skins on their (poly)phenolic composition was developed upon two seasons, under five irrigation regimes, regarding total phenolics, flavonoids, and *ortho*-diphenols, as well as individual phenolic compounds analyzed by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD). As functional tests, extracts were assessed on their radical scavenging activity *in vitro* and reducing power, and screened on their antimicrobial activity against multidrug resistant bacterial pathogens. The phenolic profile and antioxidant activities were evaluated in blancing water as well. Naringenin-7-*O*-glucoside and isorhamnetin-3-*O*-rutinoside were the most abundant phenolics in almond hulls and skins. Influence of irrigation treatments and season on phenolic content differed among by-products; hulls being more influenced by irrigation and skins by the agro-climatic conditions. The synthesis of individual phenolics was more influenced by season than treatment. According to the chemical and biological correlations, the presence of (poly)phenols seems to be responsible for the antioxidant and antimicrobial properties revealed. The knowledge generated upon the present work contributes to understand the variability of almond by-products composition attributable to seasonal and irrigation conditions, and to envisage valorization alternatives for these under explored residues and blanching water.

Keywords: almond residues; seasonal and irrigation variability; phenolic compounds; radical scavenging; reducing power; antibacterial potential

1. Introduction

Almond (*Prunus dulcis* (Mill.) D. A. Webb) has been in the last 10 years, the most produced tree nut worldwide (International Nut and Dried Fruit Council, 2016). This crop is frequently established under rainfed conditions in semi-arid locations; however, to ensure high and regular productivity, and to reach the uppermost crop's potential, the identification and use of beneficial agronomical practices and irrigation patterns constitutes a priority. The world almond production keeps following an increasing trend, achieving in the season 2016/2017 almost 1.2 M tones on the kernel basis, which is addressed to their consumption as raw nuts and in the preparation of a number of manufactured products (in chocolates, cookies, marzipan; prepared as almond butter, almond milk, etc.). This production level is 25.0% higher than the last 10 years' average (International Nut and Dried Fruit Council, 2016), while this rising production of almond by-products is enclosed to a parallel augment, which impact negatively not only on the environment of the local areas, but also on the economic balance of the industries responsible for their processing towards no-pollutant residues. In this frame, the identification of the actual biochemical profile of these materials as well as its functionality will give rise in the short term to an efficient utilization of these materials through new valorization alternatives, contributing to the "zero waste economy".

Almond production entails the generation of amounts of solid (hulls, shells, and skins) and liquid (blanching water) by-products that are mainly addressed to cattle feeding and the production of biofuels, regarding hulls and shells (Pasqualone et al., 2018), while blanched skins and blanching water usually represent, merely, a waste without further identified applications, that are mainly disposed with the consequent environmental damage. Nevertheless, in the last years these materials have been

identified as a sustainable source of valuable bioactive phytochemicals (mainly phenolic acids, flavonoids, and hydrolysable and condensed tannins) (Prgomet et al., 2017).

Developing additional research prone to shed some light on the role of these materials as sustainable sources of phenolic compounds remains pertinent because of the potential biological benefits expected from them. In this regard, previous studies on the biological properties of phenolic compounds have shown their interest for the prevention of oxidative stress and inflammation (Ferreira et al., 2017); while some authors have described almond skins on their prebiotic, antimicrobial, anti-inflammatory, and neuroprotective properties (Mandalari et al., 2011, 2010a, 2010b).

(Poly)phenols are highly influenced by diverse causes, variations concerning the phenolic content of almond skins already being described based on different factors: variety (Barreira et al., 2010), industrial processing and storage (Bolling et al., 2010a; Garrido et al., 2008; Pasqualone et al., 2018), agro-climatic conditions corresponding to distinct years (Bolling et al., 2010b), and while low or almost none attention has been dedicated to the influence of the agronomical management practices (such as irrigation strategies).

According to these antecedents, the aim of this study was to characterize the phenolic composition of almond (var. 'Ferragnès') hulls and skins, obtained upon two consecutive seasons (2015 and 2016), regarding total phenolics, flavonoids, and *ortho*-diphenols, as well as individual phenolic compounds analyzed by HPLC-DAD. To achieve this objective, the almond by-products obtained applying different irrigation regimes were studied. As functional test, (poly)phenolic extracts were assessed on their *in vitro* radical scavenging activity (ABTS and DPPH tests) and ferric reducing ability of plasma (FRAP test), as well as screened on their antimicrobial potential by the disc diffusion method against the gastrointestinal pathogens *Pseudomonas aeruginosa*,

Listeria monocytogenes, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Furthermore, blanching water was assessed on its phenolic profile and radical scavenging activity.

2. Material and methods

2.1. Experimental design and sampling

The present work was carried out on solid almond by-products (hulls and skins), and blanching water. Plant material was collected from the 15-years old almond orchard in *Alfândega da Fé*, Portugal (lat. 41°21'N; long. 6°56'W; 576 m altitude above sea level), located in the Trás-os-Montes region (North of Portugal), during two consecutive seasons (2015 and 2016). The air temperature (°C), precipitation (mm), and relative humidity (%) in the almond orchard were recorded from an automatic weather station placed in the orchard (Table 1).

Based on crop evapotranspiration (ETc), five irrigation treatments were applied: 100%, 70%, and 35% (applying the 100%, 70%, and 35% of ETc, respectively), 100-35% (100% ETc during fruit development, reducing the application down to 35% ETc during fruit filling) and 0% (rainfed; representative of normal and semi-arid conditions characteristic of the Mediterranean Basin). The weekly ETc was determined considering both the reference ET (by means of the Penman-Monteith method, using the meteorological data recorded in an automated weather station) and the crop coefficient, which ranged from 0.85 to 0.90, according to Allen et al. (1998). Within the orchard, all trees received the same annual amount of fertilizers: 66 kg N, 45 kg P_2O_5, and 45 kg K_2O per ha.

For each treatment, healthy almonds were harvested from 12 trees, located at the distinct points of the orchard. Almond hulls were separated manually from the rest of

130 the almond fruit and freeze-dried. The rest of the fruit (kernels still in shells) were left
131 air drying at room temperature, and the outer wooden shells were removed using a
132 nutcracker. Afterwards, kernels were blanched using the methodology described by
133 Milbury et al. (2006), with minor modifications, miming industrial processing; 100 g of
134 almond kernels with skin were introduced into 175 mL of deionized boiling water
135 (~95ºC), for 3 min. Skins were removed manually and oven-dried at 60°C until constant
136 weight.
137
138 *2.2. Processing of plant material and preparation of (poly)phenolic extracts*
139 Dried samples were grounded to a fine powder, and stored protected from light, at
140 room temperature, until analysis. For the extraction of phenolic compounds, samples
141 (50 mg) were mixed with 1.5 mL of methanol/distilled water (70:30, v/v), vortexed, and
142 agitated at room temperature (RT) for 30 min. Then, the mixture was centrifuged for 10
143 min at 2291 *g*, at 4ºC (Sigma 2-16K, Germany). Supernatants were collected into a 5
144 mL volumetric flask. This extraction was repeated twice more, and supernatants from
145 successive extractions were collected together. Final volume was made up to 5 mL with
146 methanol/distilled water (70:30, v/v). Extracts were then filtered through 0.45-µm
147 PVDF filters (Millex HV13, Millipore, Bedford, MA, USA) and stored at -20ºC until
148 spectrophotometric and chromatographic analyses. For the analysis of the antimicrobial
149 activity, phenolic extracts were further evaporated, freeze-dried and stored at -20ºC. To
150 apply phenolic extracts obtained from both solid almond residues to the discs for
151 antimicrobial susceptibility test, dried residues of the extracts were dissolved in 10.0%
152 dimethyl sulfoxide (DMSO).
153 Blanching water was processed as described by Mandalari et al. (2013). After
154 blanching, water was left to cool down to room temperature while stirring in an orbital

155 shaker (Model 501, Bibby Stuart, United Kindom). Fifty (50) mL were centrifuged
156 twice during 5 min, at 4°C, and 1466 *g* and the supernatant was dried using a rotary
157 evaporator (BÜCHI 461 water bath REIII, Thermo Fisher Scientific, Lisbon, Portugal).
158 Residues obtained were stored at -20°C until analysis.
159
160 *2.3. Total phenolics,* ortho*-diphenols, and flavonoids*
161 The content in total phenolics, flavonoids, and *ortho*-diphenols was determined
162 according to spectrophotometric methodologies previously described by Machado et al.
163 (2017).
164 The content of total phenolics was evaluated by the Folin Ciocalteu method, and
165 the absorbance was recorded at 750 nm. The content of *ortho*-diphenols was determined
166 by adding 40 μL of Na_2MoO_4 (50 g/L) at 160 μL of the samples appropriately diluted.
167 Mixtures were allowed to stand at room temperature, protected from light, for 15 min.
168 The absorbance was recorded at 375 nm and quantified using gallic acid as standard.
169 Results were expressed as milligrams of gallic acid equivalents per gram of dry weight
170 (mg GAE/g DW).
171 For the assessment of solid almond residues and blanching water on the content of
172 flavonoids, 24 μL of sample properly diluted were mixed with 28 μL of $NaNO_2$
173 (50 g/L). After exactly 5 min, 28 μL $AlCl_3$ (100 g/L) were added and the mixture was
174 allowed to react for 6 min. Then, 120 μL of 1 M NaOH were added. The absorbance
175 was immediately recorded at 510 nm, and the flavonoid content was quantified using
176 catechin as standard. Results were expressed as mg of catechin equivalents per gram of
177 dry weight (mg CAT/g DW).

All spectrophotometric assays were performed using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite M200 microplate reader (Tecan, Grödig, Austria). For all analyses, three replicates (n=3) of each sample were determined.

2.4. (Poly)phenolic composition by HPLC-DAD/UV

The assessment of almond by-products on their (poly)phenolic composition was performed using a HPLC-DAD/UV system equipped with a C18 column (250 × 4.6 mm, 5 µm) (ACE®-HPLC columns, Advanced Chromatography Technologies, Ltd., Aberdeen, Scotland), an eluent composed by 0.1% of trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) upon the linear gradient scheme (t in min; %B): (0; 0.0%B), (5; 0.0%B), (20; 20.0%B), (35; 50.0%B), (40; 100.0%B), (45; 0.0%B), and (65, 0.0%B). The flow rate and the injection volume were 1.0 mL min^{-1} and 10 µL, respectively. The individual phenolic acids were tentatively identified and quantified resorting to retention time, UV spectra, and UV max absorbance bands, and through comparison with authentic standards (Extrasynthese, CEDEX, France, and Sigma-Aldrich, Tauferkichen, Germany), as well as by comparing with data available in the literature (Smeriglio et al., 2016). The standards were freshly prepared in distilled water/methanol (30:70, v/v) at a concentration of 1.0 mg/mL and run in HPLC-DAD/UV simultaneously with samples. The results were expressed as micrograms per gram of dry weight (µg/g DW) for solid by-products and per liter of blanching water (µg/L).

2.5. Radical scavenging activity

The free radical scavenging activities were determined by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity, and FRAP methods, adapted to a microscale (Barros et al., 2014;

Bolanos De La Torre et al., 2015; Mena et al., 2011). The assays were performed using 96-well microplates and an Infinite M200 micro-plate reader. Briefly, for the DPPH assay, it was measured the variation in absorbance at 520 nm, after 15 min of reaction of the phenolic compounds with DPPH$^{\bullet}$, by adding 190 µL of the DPPH solution (8.87 mM) to 10 µL of sample (or standard). For ABTS the reaction consisted in mixing 188 µL of ABTS stock solution and 12 µL of sample. In respect to FRAP, 20 µL of sample followed by 280 µL of FRAP working solution were added to the 96-well microplate, and the reaction was left to incubate at 37°C for 30 min before reading the absorbance at 593 nm. All analyses were done in triplicate (n=3) for each sample. The results were expressed as millimoles of Trolox equivalents per gram of dry weight (mmol TE/g DW).

2.6. Antimicrobial exploratory approach by disc diffusion method

For the assessment of (poly)phenolic extracts on their antimicrobial activity, two extracts of hulls and two extracts of skins were taken, corresponding to these obtained under rainfed growing conditions since rainfed almond orchards are still common in many Mediterranean countries. The rational of the selection made was based on the highest phenolic content, antioxidant activity, and reducing power of such extracts that would be much informative on the actual scope of the antimicrobial activity of (poly)phenolic extracts of almond by-products.

2.6.1 Bacterial isolates and antimicrobial activity

Gram negative (*E. coli* MJS260 and *K. pneumoniae* MJS281) and Gram positive (*S. aureus* MJS241 and *E. faecalis* MJS257) bacterial isolates were collected from human faecal material in the Hospital Center of Vila Real, Portugal (CHTMAD), and

isolated and identified by the standard biochemical classification technique, according to the previously described procedure (Jorgensen et al., 1999), followed by the genetic identification over 16S rRNA sequencing. *P. aeruginosa* and *L. monocytogenes* strains were obtained from the American Type Culture Collection (ATCC). Prepared isolates were freshly inoculated in Petri dishes with Brain Heart Infusion (BHI) agar medium and incubated overnight at 37°C.

Isolated colonies were inoculated to 1.0 mL of 0.9% NaCl solution, and inoculums were further prepared by adjusting the turbidity to the 0.5 McFarland standard units. Once adjusted, suspensions were spread, with a sterile cotton swab, into Petri dishes previously filled with 20 mL of Mueller-Hinton agar (Oxoid, Basingstoke-Hampshire, UK). Sterile filter paper discs (6 mm) (Oxoid, Basingstoke-Hampshire, UK) were impregnated with 10 μL of 20 mg/mL phenolic extracts, placed on the agar plate, and plates were left to incubate during 24 h at 37°C. A negative control (10 μL of solvent, 10.0% DMSO) and three (3) positive controls (the commercial antibiotics gentamicin (Oxoid, Basingstoke-Hampshire, UK) in two different concentrations (10 and 30 μg per disc) and ciprofloxacin (10 μg per disc) (Oxoid, Basingstoke-Hampshire, UK)) were used.

After incubation during 24 h, the diameter of halos (growth inhibition zones) in mm was recorded. Antimicrobial activity was expressed as an average of inhibition zone diameters (mm) and as a percentage of relative inhibition zone diameter (%RIZD) according to Aires et al. (2009) by the application of the equation '*%RIZD=((IZD sample – IZD negative control) / IZD antibiotic standard) x 100*', where IZD is inhibition zone diameter (mm). According to that, antibacterial effects were classified based on the following activity score (Gouvinhas et al., 2018): 0 – without effect; 0-100 – less effective than an antibiotic; >100 – more effective than an

antibiotic; ¤ □ - extract effective and antibiotic without effect. All tests were performed in triplicate (n = 3).

2.7. Statistical analysis

The results are presented as means (n=3) with the determination of the Least Significant Differences (LSD) for a p value <0.05. The data were subjected to two-way analysis of variance (ANOVA) and a multiple range test (Tukey's test), using Statgraphics Centurion XVI (StatPoint Tecnhologies, Inc., 2010, USA). Pearson correlation analysis was performed to corroborate relationships between selected parameters.

3. Results and discussion

3.1. Almond by-products' total phenolics, ortho-*diphenols, and flavonoids*

The content of total phenolics, *ortho*-diphenols, and flavonoids of almond by-products is shown in the Figure 1. The total phenolic content of blanching water was in the range 392.16-505.95 mg GAE/L, while in freeze-dried hulls and blanched and oven-dried skins were in the ranges 7.90–32.66 and 13.44–34.71 mg GAE/g DW, respectively. Regarding flavonoids and *ortho*-diphenols, blanching water exhibited the following ranges of concentration: 292.78–467.78 mg CAT/L and 224.21–318.07 mg GAE/L, respectively. On the other hand, skins (11.14–34.43 mg CAT/g DW and 10.65–26.59 mg GAE/g DW, respectively) surpassed the concentrations recorded for hulls (4.28–29.05 mg CAT/g DW and 8.28–24.53 mg GAE/g DW, respectively).

When comparing among the diversity of irrigation levels, significant differences were observed for each material considered separately. The phenolic content present in skins appeared more influenced by the season, while a lower effect of the different

278 irrigation treatments assayed in the present work was observed. Garrido et al. (2008)
279 and Bartolomé et al. (2010) have previously demonstrated that total phenolic content of
280 almond skin from mixtures of Spanish and American varieties, obtained upon diverse
281 seasons, varied between 9.10 and 32.10 mg GAE/g DW, with differences of almost 35%
282 between seasons 2004 and 2006. In the present study seasonal variation in the total
283 phenolic content was observed between 15.0 and 55.0% for almond skins, depending on
284 the irrigation treatments applied, thus pointing up the seasonal variability as a critical
285 factor of the total phenolic content present in almond by-products. Additionally, Bolling
286 et al. (2010b) have reported the effect of cultivar and harvest year (and the associated
287 diverse agro-climatic conditions) on the concentration of (poly)phenols in almond skins,
288 both factors having significant impact. Indeed, that report stressed the need of further
289 studies on controlled agronomical practices to identify those factors responsible for the
290 differences described. In this regard, the data obtained in the present work support the
291 hypothesis of significance of irrigation effect and seasonal variability, for almond by-
292 products.

293 The content of total phenolics in hulls and blanching water differed significantly
294 among irrigation treatments, although both matrices were featured by a reverse trend
295 (Figure 1). Hence, in hulls the highest concentration of total phenolics, *ortho*-diphenols,
296 and flavonoids corresponded to not irrigated trees (27.11 and 19.98 mg GAE/g DW, and
297 22.52 mg CAT/g DW, on average, respectively) that exhibited significantly higher
298 concentrations than samples corresponding to trees irrigated with 100% and 70% ETc
299 (16.45 and 13.60 mg GAE/g DW, and 14.34 mg CAT/g, on average, respectively).
300 Thus, the effect of the diverse irrigation regimes regarding the concentration of phenolic
301 compounds in hulls was highly significant (p<0.001) and stronger compared with the
302 effect of the agro-climatic conditions recorded in each season (2015 and 2016). This

303 evidenced a consistent tendency in both seasons (Figure 1) through which an increase of
304 the irrigation water applied lowered significantly the phenolic content in hulls.
305 According to this result, higher concentrations found in hulls of rainfed trees were
306 probably due to a (poly)phenolic accumulation in response to environmental stress, in
307 agreement with the already described role of these molecules in higher plants.
308 Concerning this, the outer part of almond fruit, where the most extreme variation
309 regarding irrigation regimes was observed, might be more subjected to stress conditions,
310 such as drought (Mouradov and Spangenberg, 2014).

311 Apart from the seasonal agro-climatic influence, Sfahlan et al. (2009) have
312 reported differences between genotypes regarding the phenolic content of almond hulls,
313 reporting concentrations much higher that ranged between 36 and 167 mg GAE/g of
314 extract, while Barreira et al. (2010) compared hulls of different almond varieties on total
315 phenolic and flavonoid content, including the variety used in this study ('Ferragnès')
316 and a production area (North of Portugal) featured by equal Continental agro-climatic
317 conditions. However, their results were around up to 10-folds higher than the herein
318 presented ones (130.68 and 378.00 mg/g for flavonoids and total phenolics,
319 respectively). These differences, however, together with the different climatic
320 conditions featuring seasons in which the field experiments were developed, further
321 stress the importance of samples processing, extraction methods, harvest time, and
322 irrigation strategies.

323 Interestingly, when analyzing the variation of the content of total phenolics,
324 *ortho*-diphenols, and flavonoids in blanching water, the highest level corresponded to
325 water obtained from blanching almonds of full irrigated plants (100%) (458.63 and
326 290.59 mg GAE/L and 406.98 mg CAT/L, on average, respectively), these
327 concentrations decreasing with the reduction of water supply, and surpassing the level

recorded in blanching water from processing material of not irrigated plants by 8.3, 13.1, and 12.7%, respectively (Figure 1). These results should be seen as a whole together with almond skins, as all the (poly)phenols found in blanching water are lost from almond skins during the blanching process. Even though the almond skin was featured with similar phenolic content compared with ~~to~~ hulls, 94.0% of phenolics was lost to blanching water from skins in the current study, what is in agreement with previous studies that reported 74.0-88.0% loss of phenolics in water during blanching (Milbury et al., 2006). The high temperature of water had probably increased the extraction of phenolics from skin to the blanching water as the total phenolic content were around 15 times greater in the latter one. This fact was already described by Hughey et al. (2012) who noticed that total phenolics in blanching water could be up to 50-times greater than in blanched skins. These findings reinforce the features of blanching water as a rich source of (poly)phenols, independently of the almond variety and seasons. However, even so, according to the antimicrobial and antioxidant properties of blanched skins associated to the phenolic composition that remains after blanching (Mandalari et al., 2010), this by-product should be further considered a valuable source of functional compounds with antimicrobial and antioxidant properties.

3.2. Changes in (poly)phenolic profile of almond by-products under irrigation regimes during two seasons

The HPLC analysis of almond hulls and skins, as well as blanching water, revealed a wide variety of phenolic compounds (Tables 2-4) belonging to different phenolic classes.

Concerning phenolic acids belonging to benzoic acids, in 2015, it was observed the presence of protocatechuic and *p*-hydroxybenzoic acids in both skins and blanching

353 water (Tables 3-4) in agreement with Pasqualone et al. (2018), while in hulls only
354 protocatechuic acid was detected (Table 2). The highest concentration of protocatechuic
355 acid in hulls and skins was recorded in material collected under 100% irrigation
356 condition (3.15 and 1.36 μg/g DW) that surpassed significantly the concentration in
357 material from the remaining conditions by 18.7% (hulls) and 22.6% (skins), on average.
358 In blanching water, the highest concentration of protocatechuic acid was obtained after
359 processing material from non-irrigated trees (28.31 μg/L), while water from processing
360 material under other irrigation conditions remained in similar lower levels (17.11 μg/L).
361 On the other hand, the highest concentration of *p*-hydroxybenzoic acid in skins was
362 observed in materials from trees irrigated at 35% (2.99 μg/g DW), followed by 100%
363 and 100%-35% (2.62 μg/g DW, on average), 70% (1.71 μg/g DW), and non-irrigated
364 (1.31 μg/g DW) (Table 3). In blanching water, the highest concentration was observed
365 when no irrigation was applied, and in water used to process material from almonds
366 exposed to 35% and 100%-35% irrigation conditions (5.96 μg/L, on average) (Table 4).
367 In respect to the cinnamic acids, it was revealed the occurrence of chlorogenic and
368 *trans*-*p*-coumaric acids (Tables 2-4), both present in hulls, being highlighted 35%
369 irrigation as the most appropriate condition for the occurrence of these compounds in
370 hulls (11.55 and 3.34 μg/g DW, respectively) (Table 2). However, in skins and
371 blanching water, only *trans*-*p*-coumaric and chlorogenic acids, respectively, were found
372 in quantifiable levels (Tables 3 and 4). The optimal irrigation conditions for the
373 occurrence of both compounds differed. While the highest level of *trans*-*p*-coumaric
374 acid in skins was retrieved from almonds exposed to 100% irrigation conditions
375 (1.34 μg/g DW) (Table 3), in respect to blanching water, the maximum concentration of
376 chlorogenic acid exhibited an erratic behavior, being detected under no irrigation, as
377 well as at 70 and 100% irrigation rates (Table 4).

When analyzing the level of phenolic acids in samples obtained upon the 2016 season, in hulls, equal relative abundance of protocatechuic, chlorogenic, and *trans*-*p*-coumaric acids was found (Table 2). In addition to these phenolics, it was observed the presence of *p*-hydroxycinnamic (5.67 μg/g DW) and vanillic acids (2.32 μg/g DW), which highest abundance corresponded to rainfed trees and trees irrigated with 100% ETc, respectively (Table 2).

Hulls are already known to be a rich source of phenolic acids, and in specific, of chlorogenic (Takeoka and Dao, 2003) and protocatechuic (Sang et al., 2002b) acids. As demonstrated herein, the presence of protocatechuic, *p*-hydroxybenzoic, and trans-*p*-coumaric acid (Arráez-Román et al., 2010; Bolling et al., 2009) was earlier already reported, however, no studies, to the best of authors' knowledge, up to date, report the irrigation variability of phenolic acids in almond by-products.

When evaluating the separate classes of phenolic compounds present in the matrices under study, flavonoids, which are products of the shikimate pathway closely affected by the water supply regime (Koh and Mitchell, 2008), were represented by a plethora of individual compounds including up to seven flavonoids (naringenin-7-*O*-glucoside, eriodictyol-7-*O*-glucoside, (-)-epicatechin, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, and isorhamnetin). In detail, in hulls collected in both 2015 and 2016 seasons was found the presence of naringenin-7-*O*-glucoside (19.62-105.56 μg/g DW), kampferol-3-*O*-rutinoside (0.37-1.73 μg/g DW), kampferol-3-*O*-glucoside (0.34-1.19 μg/g DW), isorhamnetin-3-*O*-rutinoside (1.84-7.96 μg/g DW), isorhamnetin-3-*O*-glucoside (0.37-0.92 μg/g DW), and ishorhamnetin (0.48-1.31 μg/gz DW) (Table 2). On the other hand, flavonoids identified in skin extracts were naringenin-7-*O*-glucoside (1.50-10.95 μg/g DW), eriodictyol-7-*O*-glucoside (1.05-2.64 μg/g DW), (-)-epicatechin (2.49-4.44 μg/g DW), kaempferol-3-*O*-

glucoside (1.35-1.96 μg/g DW), isorhamnetin-3-*O*-rutinoside (5.43-7.10 μg/g DW), isorhamnetin-3-*O*-glucoside (0.87-1.90 μg/g DW), and isorhamnetin (1.40-2.32 μg/g DW) (Table 3). Similar profiles were previously reported by several authors (Bartolomé et al., 2010; Bolling et al., 2010a; Pasqualone et al., 2018), that have identified the major flavonoids in almond skin extracts. However, seasonal variation seems to be not linear for different irrigation regimes. Hence, (-)-epicatechin present in almond skin was reported by Bolling et al. (2010b) as the only phenolic compound that varies significantly between seasons (1.7-folds, on average from 2005 to 2007). Besides, Martinez et al. (2016) developed an exhaustive metabolomic study that revealed the high dependency of the accumulation of phenolic compounds on the type of abiotic stress applied. Probably, a combination of diverse climatic conditions with specific irrigation treatments influenced the phenolic composition profile and concentration of herein studied almond by-products.

The major difference between seasons was observed for the naringenin-7-*O*-glucoside, compound that was predominant in almond skins and hulls, where in 2016 was noticed a general decrease, regardless the treatment, of around 3-folds compared with 2015 in both almond skins and hulls. Interestingly, this flavanone was always present in lower concentrations in rainfed treatment compared with all the others and was not detected in blanching water. The most abundant flavonoid in blanching water was isorhamnetin-3-*O*-rutinoside (60.53-80.33 μg L^{-1} DW), followed by eriodictyol-7-*O*-glucoside (24.32-34.35 μg L^{-1} DW), and kampferol-3-*O*-glucoside (15.72-20.68 μg L^{-1} DW) (Table 4). Isorhamnetin-3-*O*-glucoside, isorhamnetin, naringenin-7-*O*-glucoside, *trans*-p-coumaric acid, and (-)-epicatechin were not detected in blanching water. Several authors (Hughey et al., 2012; Mandalari et al., 2013, 2010c; Smeriglio et al., 2016) have reported similar qualitative profile as the one from the current study, although direct

comparisons are not possible due to the diverse origins, extraction methods, varieties, and other conditions. In this regard, while some authors did not detect naringenin-7-*O*-glucoside nor in skin neither in blanching water (Smeriglio et al., 2016), others reported this compound to be present in almond skins (Arráez-Román et al., 2010) and/or in blanching water in high concentrations (Mandalari et al., 2010a; Pasqualone et al., 2018). Still, the high concentration of the individual phenolics detected in this almond by-product contributes to confirm blanching water as a possible source of these bioactive compounds.

3.3. Radical scavenging activity, reducing power, and antimicrobial potential of industrial almond by-products phenolics

When analyzing the differential radical scavenging capacity between the separate residues obtained from the range of irrigation regimes assayed, in agreement with the information retrieved on the content in total phenolics, blanching water displayed a considerably high activity (3.05-3.41 mmol TE/L for ABTS and 1.98-2.17 mmol TE/L for DPPH), as well as regarding the reducing power (3.10-3.64 mmol TE/L). Besides, hulls and skins remained in somehow lower levels of radical scavenging activity (0.10-0.16 and 0.16-0.18 mmol TE/g DW for ABTS, 0.07-0.12 and 0.10-0.11 mmol TE/g DW for DPPH, respectively) and reducing power (0.18-0.28 and 0.06-0.07 mmol TE/g DW, respectively). Among treatments, hulls obtained from non-irrigated trees were featured by the strongest activity, while the lowest corresponded to 70% and 100% ETc. For skins results were less uniform, although, in general following an equal pattern.

Many studies have focused the antioxidant activity of almond by-products by radical scavenging methods (DPPH and ABTS) (Chen and Blumberg, 2008; Mandalari et al., 2010c; Monagas et al., 2009; Pasqualone et al., 2018; Sang et al., 2002a;

453 Smeriglio et al., 2016), and the method developed to monitor the reducing power
454 (FRAP) (Bolling et al., 2010b; Chen and Blumberg, 2008; Smeriglio et al., 2016). These
455 works have contributed to demonstrate the interest of these matrices as valuable
456 materials containing a high phenolic composition with valuable functional features. As
457 the synthesis of (poly)phenolics have been associated with a response to abiotic stress
458 (indeed, these are considered secondary metabolites of response to stress), higher
459 antioxidant capacities were expected from materials obtained from trees exposed to
460 irrigation deficit.

461 Following these results, antimicrobial activity was assessed for the phenolic
462 extracts of hulls and skins, in both years for samples from the rainfed treatment.
463 (Poly)phenols play an important role in enhancing the antimicrobial potential against
464 multidrug resistant bacteria (Daglia, 2012). As expected, the negative solvent (10.0%
465 DMSO) used for the extract's preparation was not effective against the target
466 gastrointestinal bacteria. Extracts of skins corresponding to the 2016 season exhibited
467 the strongest growth suppression for *L. monocytogenes*, *S. aureus*, *E. faecalis*, and *P.*
468 *aeruginosa* (10.67, 11.33, 11.67, and 11.33 mm, respectively), followed by hulls
469 obtained in the season 2015 (9.33, 10.33, 10.33, and 10.00 mm, respectively), skins of
470 the season 2015 (9.00, 9.67, 9.67, and 9.33 mm, respectively), and hulls of the season
471 2016 (8.33, 8.67, 9.00, and 9.33 mm, respectively). However, (poly)phenolic extracts of
472 almond by-products were not able to inhibit *E. coli* and *K. pneumoniae* growth. On the
473 other side, all four extracts showed antimicrobial activity against *S. aureus* and *E.*
474 *faecalis*, reaching higher efficiency than medical antibiotics included in this study as
475 positive controls, as antibiotics did not show any effect against those bacteria. These
476 antibiotics (ciprofloxacin and gentamicin) are featured by a broad range of antibacterial
477 activity, ciprofloxacin being used as broad-spectrum antibiotic against both Gram-

positive and Gram-negative bacteria, while gentamicin is used to treat several types of bacterial infections, mostly Gram-negative bacteria, including *P. aeruginosa*, *E. coli*, and *K. pneumoniae*.

Antimicrobial activity expressed as %RIZD (relative inhibition zone diameter) provides a broader picture on the effect of the extract compared with positive controls (clinical antibiotics). Values of %RIZD for ciprofloxacin (Table 5) in the present study varied from 40.0 to 52.0% for *L. monocytogenes* and from 47.0 to 57.0% for *P. aeruginosa*, evidencing that extracts are as half as effective as the ciprofloxacin at the tested dose. The %RIZD for gentamicin ranged from 97.0 to >100.0 % for *P. aeruginosa* at the lowest dose, and from 76.0 to 92.0% at the highest one, while for *L. monocytogenes* ranged from 44.0 to 56.0% at the lower dose and from 40.0 to 52.0% at the higher one (Table 5). When tested against *S. aureus* and *E. faecalis*, phenolic extracts in this study exhibited a higher effectiveness than the antibiotics, as antibiotics were not effective at all (Table 5). Mandalari et al. (2010a) and Smeriglio et al. (2016) have reported the phenolic fraction of almond skins to have significant antimicrobial activity against the Gram positive bacteria strains *L. monocytogenes* and *S. aureus*. These antimicrobial properties were attributed to the dominant phenolics present in extracts: (-)-epicatechin, protocatechuic acid, isorhamnetin-3-*O*-rutinoside, naringenin-7-*O*-glucoside, as previously demonstrated regarding the activity of almond skins phenolics against food-borne pathogens individually as well as dedicated combinations of protocatechuic acid, naringenin, and epicatechin (Mandalari et al., 2010a). Regarding efficiency, naringenin was identified as the most efficient compound concerning antimicrobial power, followed by epicatechin, protocatechuic acid, catechin, and isorhamnetin-3-*O*-glucoside. These compounds were also identified in the phenolic extracts evaluated in the present study were noticed as responsible, in a significant

503 extent, for the observed antimicrobial activity. In this regard, resorting to the analysis of
504 correlation between the concentration of the individual phenolics in the (poly)phenolic
505 extract with the antimicrobial activity found. Hence, positive significant correlation was
506 found against *P. aeruginosa* in comparison with Gentamicin (30 µg/disc) and
507 Ciprofloxacin (10 µg/disc) for (-)-epicatechin ($r=0.999^{p<0.001}$ and $r=0.919^{p<0.05}$,
508 respectively), isorhamnetin-3-*O*-glucoside ($r=0.999^{p<0.01}$ and $r=0.989^{p<0.01}$, respectively),
509 and eridictyol-7-*O*-glucoside ($r=0.976^{p<0.05}$ and $r=0.976^{p<0.05}$, respectively). In addition,
510 according to significant positive correlation, the individual phenolic compounds in skins
511 responsible for the inhibition against *L. monocytogenes* in comparison with Gentamicin
512 (30 µg/disc) and Ciprofloxacin (10 µg/disc) were eridictyol-7-*O*-glucoside
513 ($r=0.918^{p<0.05}$ and $r=0.999^{p<0.05}$, respectively) and naringenin-7-*O*-glucoside
514 ($r=0.909^{p<0.05}$ and $r=0.685^{p<0.05}$, respectively). In hulls, compound positively and
515 significantly correlated with antimicrobial activity against *P. aeruginosa* was
516 isorhamnetin-3-*O*-rutinoside ($r=0.993^{p<0.05}$), while against *L. monocytogenes* was
517 naringenin-7-*O*-glucoside ($r=0.999^{p<0.05}$). FOR ME, IT IS NOT CLEAR THE
518 PRESENTATION OF THE CORRELATION COEFFICIENTS BY COMPARING
519 WITH THE ANTIBIOTICS ?? TWO CORRELATIONS FOR A SINGLE
520 COMPOUNDS ?? MAY BE BECAUSE YOU PRESENT THE ANTIMICROBIAL
521 ACTIVITY RELATIVELY TO THE ACTIVITY OF THE CLINICAL ANTIBIOTICS
522 ??

523 Mechanisms of action of (poly)phenols occurring could be different; however, the
524 most probable one is identified as the aggregatory effect on the bacterial cells (Daglia,
525 2012). (Poly)phenols can link to bacterial cells membrane, disturbing the membrane
526 function and inhibiting the cell growth (Cardona et al., 2013). On the other side, for *E.*
527 *coli* and *K. pneumoniae*, extracts did not suppress the bacterial growth at any level,

528 therefore the %RIZD being 0. These occurrences might be because of the differences in
529 the wall composition of Gram-positive and Gram-negative bacteria, Gram-negative
530 bacteria exhibiting higher resistance to (poly)phenols (Cardona et al., 2013).

531 Even though the current study is focused on screening the antimicrobial activity of
532 almond hulls and skins, additional experiments should be performed that might provide
533 complementary information for design of new antibiotics and/or use of these by-
534 products as natural antimicrobial agents with medical or technological applications, as
535 multidrug resistance is nowadays a norm among these pathogens (Lowy, 2003). In this
536 aspects, recently it has been suggested that combining bioactive (poly)phenols with
537 antibiotics may lead to equal or even enhanced antimicrobial effect, while using lower
538 doses of (poly)phenols/antibiotics and thus, excluding eventual side effects associated to
539 the medical treatments. Furthermore, the use of phenolic extracts of almond by-products
540 as, for instance, food additives, would include the compounds' isolation, purification,
541 stabilization, and incorporation to newly developed food products, with a purpose of
542 antimicrobial protection, but with no effect on the sensorial and nutritional properties,
543 and on health security (Martillanes et al., 2017). On the other side, active packaging
544 used in food industry with incorporated phenolic compounds present in the residues of
545 the almond production and processing would contribute to improve the sensory
546 properties, prolong shelf life, and maintain the quality of raw and processed foods
547 (Martillanes et al., 2017). In addition, phenolic extracts used for the production of
548 functional foods and dietary supplements, are commonly encapsulated, and this could
549 constitute a practical solution to avoid degradation of (poly)phenols, ensure compounds'
550 stability, and enhance the shelf life of this type of bioactive phytochamicals (Brglez et
551 al., 2016; Munin and Levy, 2011). These further steps towards to practical applicability
552 of phenolic extracts from almond by-products could lead not just to creation of

553 innovative products but, at the same time, would contribute to the reduction of the
554 environmental impact, by valorizing the waste from the almond production chain.
555
556 *3.4. Almond by-products phenolics correlation to antioxidant activities*
557 Plant phenolics is a group of compounds that are featured by free radical
558 scavenging activities (Kulbat, 2016). Hence, as expected, the total phenolic content in
559 the present study was positively and highly significantly correlated to the result
560 retrieved from the antioxidant activity and reducing power determinations. These
561 findings support results previously reported in the literature regarding diverse parts of
562 the almond fruit (Barreira et al., 2009; Isfahlan et al., 2010). Additionally, in hulls,
563 positive and significant correlations were observed between chlorogenic acid,
564 isorhamnetin-3-*O*-glucoside, and isorhamnetin concentrations with antioxidant activities
565 in extracts obtained of hulls materials collected in both years (Table 6). Likewise,
566 positive and significant correlations were observed for skins extracts between the
567 concentration of eriodictyol-7-*O*-glucoside, isorhamnetin-3-*O*-rutinose, and
568 isorhamnetin and the antioxidant activity tests (Table 6).
569 *Ortho*-diphenols are the phenolic class including compounds with two OH groups
570 in the *ortho* position of the ring due to which these compounds are responsible for the
571 high antioxidant activity of the (poly)phenolic extracts. Indeed, chlorogenic acid and
572 eriodictyol-7-*O*-glucoside are *ortho*-diphenols and their strong correlation with ABTS,
573 DPPH, and FRAP-based results can lead to these compounds as being responsible for
574 antioxidant activity of almond hulls and skins. Regarding the rest of significantly related
575 compounds, isorhamnetin displayed in all cases stronger and more significant
576 correlations to antioxidant activity assays compared with its glycosylated forms
577 (isorhamnetin-3-*O*-glucoside and isorhamentin-3-*O*-rutinoside for hulls and skins,

578 respectively), while glycosylated flavonoids are featured by lower DPPH and ABTS
579 capacities compared with their aglycones, according to structure-activity relationship
580 analyses (Csepregi et al., 2016).
581
582 **5. Conclusions**
583 Results from the current study demonstrated the occurrence of phenolics in
584 almond by-products being differently influenced by the diverse agro-climatic conditions
585 featuring separate seasons and specific irrigation patterns. This might be relevant for the
586 potential application of diverse agricultural management practices addressed to enhance
587 the bioactive (phenolic) content and thus, the beneficial properties of almond by-
588 products, allowing to draw new added-value valorization alternatives for these waste
589 materials. According to the previous description of the biological and chemical features
590 of (poly)phenols, the presence of phenolic compounds seems to be responsible for the
591 antioxidant and antibacterial properties of almond by-products. Apart from the
592 possibility of usage of these by-products due to their beneficial properties for design of
593 new antibiotics, other valorization options are emerging, such as using them as valuable
594 natural antimicrobial agents for industrial uses. All these data together further contribute
595 to better understanding of seasonal and irrigation variability, as well as to envisage new
596 valorization alternatives for solid almond residues and blanching water obtained during
597 the industrial processing.
598
599 **Acknowledgements**
600 IP acknowledges the financial support provided by the FCT-Portuguese Foundation for Science and
601 Technology (SFRH/BD/52539/2014), under the Doctoral Programme “Agricultural Production Chains –
602 from fork to farm” (PD/00122/2012). This work was also supported by: European Investment Funds by
603 FEDER/COMPETE/POCI – Operacional Competitiveness and Internacionalization Programme, under

Project POCI-01-0145-FEDER-006958 and National Funds by FCT - Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013. RDP was supported by a Postdoctoral Contract (Juan de la Cierva de Incorporación ICJI-2015-25373) from the Ministry of Economy, Industry and Competitiveness of Spain.

References

Aires, A., Mota, V.R., Saavedra, M.J., Rosa, E.A.S., Bennett, R.N., 2009. The antimicrobial effects of glucosinolates and their respective enzymatic hydrolysis products on bacteria isolated from the human intestinal tract. J. Appl. Microbiol. https://doi.org/10.1111/j.1365-2672.2009.04180.x

Allen, R.G., Pereira, L.S., Raes, D., Smith, M., 1998. Crop evapotranspiration: Guidelines for computing crop water requirements, in: FAO. p. 300. https://doi.org/10.1016/j.eja.2010.12.001

Arráez-Román, D., Fu, S., Sawalha, S.M.S., Segura-Carretero, A., Fernández-Gutiérrez, A., 2010. HPLC/CE-ESI-TOF-MS methods for the characterization of polyphenols in almond-skin extracts. Electrophoresis 31, 2289–2296. https://doi.org/10.1002/elps.200900679

Barreira, J.C.M., Ferreira, I.C.F.R., Oliveira, M.B.P.P., Pereira, J. a, 2010. Antioxidant potential of chestnut (*Castanea sativa* L.) and almond (*Prunus dulcis* L.) by-products. Food Sci. Technol. Int. 16, 209–216. https://doi.org/10.1177/1082013209353983

Barreira, J.C.M., Ferreira, I.C.F.R., Oliveira, M.B.P.P., Pereira, J.A., 2009. Effects of different phenols extraction conditions on antioxidant activity of almond (*Prunus dulcis*) fruits. J. Food Biochem. https://doi.org/10.1111/j.1745-4514.2009.00249.x

Barros, A., Gironés-Vilaplana, A., Teixeira, A., Collado-González, J., Moreno, D.A., Gil-Izquierdo, A., Rosa, E., Domínguez-Perles, R., 2014. Evaluation of grape (*Vitis vinifera* L.) stems from Portuguese varieties as a resource of (poly)phenolic compounds: A comparative study. Food Res. Int. 65, 375–384. https://doi.org/10.1016/j.foodres.2014.07.021

Bartolomé, B., Monagas, M., Garrido, I., Gómez-Cordovés, C., Martín-Álvarez, P.J., Lebrón-Aguilar, R., Urpí-Sardà, M., Llorach, R., Andrés-Lacueva, C., 2010. Almond (*Prunus dulcis* (Mill.) D.A. Webb) polyphenols: From chemical characterization to targeted analysis of phenolic metabolites in humans. Arch. Biochem. Biophys. 501, 124–133. https://doi.org/10.1016/j.abb.2010.03.020

Bolanos De La Torre, A.A.S., Henderson, T., Nigam, P.S., Owusu-Apenten, R.K., 2015. A universally calibrated microplate ferric reducing antioxidant power (FRAP) assay for foods and applications to

Manuka honey. Food Chem. https://doi.org/10.1016/j.foodchem.2014.11.009

Bolling, B.W., Blumberg, J.B., Chen, C.Y.O., 2010a. The influence of roasting, pasteurisation, and storage on the polyphenol content and antioxidant capacity of California almond skins. Food Chem. 123, 1040–1047.

Bolling, B.W., Dolnikowski, G., Blumberg, J.B., Chen, C.Y.O., 2010b. Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. Food Chem. https://doi.org/10.1016/j.foodchem.2010.03.068

Bolling, B.W., Dolnikowski, G., Blumberg, J.B., Chen, C.Y.O., 2009. Quantification of almond skin polyphenols by liquid chromatography-mass spectrometry. J. Food Sci. 74. https://doi.org/10.1111/j.1750-3841.2009.01133.x

Brglez Mojzer, E., Knez Hrnčič, M., Škerget, M., Knez, Ž., Bren, U., 2016. Polyphenols: extraction methods, antioxidative action, bioavailability and anticarcinogenic effects. Molecules, 21(7). doi:10.3390/molecules21070901

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F.J., Queipo-Ortuño, M.I., 2013. Benefits of polyphenols on gut microbiota and implications in human health. J. Nutr. Biochem. 24, 1415–1422. https://doi.org/10.1016/j.jnutbio.2013.05.001

Chen, C.Y.O., Blumberg, J.B., 2008. In vitro activity of almond skin polyphenols for scavenging free radicals and inducing quinone reductase. J. Agric. Food Chem. 56, 4427–4434. https://doi.org/10.1021/jf800061z

Csepregi, K., Neugart, S., Schreiner, M., Hideg, É., 2016. Comparative evaluation of total antioxidant capacities of plant polyphenols. Molecules. https://doi.org/10.3390/molecules21020208

Daglia, M., 2012. Polyphenols as antimicrobial agents. Curr. Opin. Biotechnol. https://doi.org/10.1016/j.copbio.2011.08.007

Ferreira, I.C.F.R., Martins, N., Barros, L., 2017. Phenolic compounds and its bioavailability: In vitro bioactive compounds or health promoters?, in: Advances in Food and Nutrition Research. https://doi.org/10.1016/bs.afnr.2016.12.004

Garrido, I., Monagas, M., Gómez-Cordovés, C., Bartolomé, B., 2008. Polyphenols and antioxidant properties of almond skins: Influence of industrial processing. J. Food Sci. 73. https://doi.org/10.1111/j.1750-3841.2007.00637.x

Hughey, C.A., Janusziewicz, R., Minardi, C.S., Phung, J., Huffman, B.A., Reyes, L., Wilcox, B.E.,

Prakash, A., 2012. Distribution of almond polyphenols in blanch water and skins as a function of blanching time and temperature. Food Chem. 131, 1165–1173. https://doi.org/10.1016/j.foodchem.2011.09.093

International Nut and Dried Fruit Council, 2016. World tree nut production. Reus, Spain.

Isfahlan, A.J., Mahmoodzadeh, A., Hassanzadeh, A., Heidari, R., Jamei, R., 2010. Antioxidant and antiradical activities of phenolic extracts from Iranian almond (*Prunus amygdalus* L.) hulls and shells. Turk J Biol. https://doi.org/10.3906/biy-0807-21

Jorgensen, J.H., Turnidge, J.D., Washington, J.., 1999. Antibacterial susceptibility tests: dilution and disk diffusion methods., in: Murray, P.R., Baron, E.J., Pfaller, M.A., Tenover, F.C., Yolken, R.. (Eds.), Manual of Clinical Microbiology. ASM Press, Washington DC, pp. 1526–1543.

Koh, E., Mitchell, A., 2008. Trends in the analysis of phytochemicals flavonoids and carotenoids, in: Meskin, M., Bidlack, W., Randolph, R.K. (Eds.), Phytochemicals. pp. 39–76. https://doi.org/https://doi.org/10.1201/9781420061383.ch3

Kulbat, K., 2016. The role of phenolic compounds in plant resistance. Biotechnol Food Sci.

Lowy, F.D., 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. J. Clin. Invest. https://doi.org/10.1172/JCI18535

Machado, N., Domínguez-Perles, R., Ramos, A., Rosa, E.A.S., Barros, A.I.R.N.A., 2017. Spectrophotometric versus NIR-MIR assessments of cowpea pods for discriminating the impact of freezing. J. Sci. Food Agric. 97, 4285–4294. https://doi.org/10.1002/jsfa.8251

Mandalari, G., Arcoraci, T., Martorana, M., Bisignano, C., Rizza, L., Bonina, F.P., Trombetta, D., Tomaino, A., 2013. Antioxidant and photoprotective effects of blanch water, a byproduct of the almond processing industry. Molecules 18, 12426–12440. https://doi.org/10.3390/molecules181012426

Mandalari, G., Bisignano, C., D'Arrigo, M., Ginestra, G., Arena, A., Tomaino, A., Wickham, M.S.. J., 2010a. Antimicrobial potential of polyphenols extracted from almond skins. Lett. Appl. Microbiol. 51, 83–89. https://doi.org/10.1111/j.1472-765X.2010.02862.x

Mandalari, G., Bisignano, C., Genovese, T., Mazzon, E., Wickham, M.S.. J., Paterniti, I., Cuzzocrea, S., 2011. Natural almond skin reduced oxidative stress and inflammation in an experimental model of inflammatory bowel disease. Int. Immunopharmacol. 11, 915–924. https://doi.org/10.1016/j.intimp.2011.02.003

694 Mandalari, G., Faulks, R.M., Bisignano, C., Waldron, K.W., Narbad, A., Wickham, M.S.. J., 2010b. In
695 vitro evaluation of the prebiotic properties of almond skins (*Amygdalus communis* L.). FEMS
696 Microbiol. Lett. 304, 116–122. https://doi.org/10.1111/j.1574-6968.2010.01898.x
697 Mandalari, G., Tomaino, A., Arcoraci, T., Martorana, M., Turco, V. Lo, Cacciola, F., Rich, G.T.,
698 Bisignano, C., Saija, A., Dugo, P., Cross, K.L., Parker, M.L., Waldron, K.W., Wickham, M.S.. J.,
699 2010c. Characterization of polyphenols, lipids and dietary fibre from almond skins (*Amygdalus*
700 *communis* L.). J. Food Compos. Anal. 23, 166–174. https://doi.org/10.1016/j.jfca.2009.08.015
701 Martillanes, S., Rocha-Pimienta, J., Cabrera-Bañegil, M., Martín-Vertedor, D., Delgado-Adámez, J.,
702 2017. Application of phenolic compounds for food preservation: Food additive and active
703 packaging. In Phenolic Compounds-Biological Activity. InTech. http://dx.doi.org/10.5772/66885
704 Martinez, V., Mestre, T.C., Rubio, F., Girones-Vilaplana, A., Moreno, D.A., Mittler, R., Rivero, R.M.,
705 2016. Accumulation of flavonols over hydroxycinnamic acids favors oxidative damage protection
706 under abiotic stress. Front. Plant Sci. https://doi.org/10.3389/fpls.2016.00838
707 Mena, P., García-Viguera, C., Navarro-Rico, J., Moreno, D.A., Bartual, J., Saura, D., Martí, N., 2011.
708 Phytochemical characterisation for industrial use of pomegranate (*Punica granatum* L.) cultivars
709 grown in Spain. J. Sci. Food Agric. https://doi.org/10.1002/jsfa.4411
710 Milbury, P.E., Chen, C.Y., Dolnikowski, G.G., Blumberg, J.B., 2006. Determination of flavonoids and
711 phenolics and their distribution in almonds. J. Agric. Food Chem. 54, 5027–5033.
712 https://doi.org/10.1021/jf0603937
713 Monagas, M., Garrido, I., Lebrón-Aguilar, R., Carmen Gómez-Cordovés, M., Rybarczyk, A., Amarowicz,
714 R., Bartolomé, B., 2009. Comparative flavan-3-ol profile and antioxidant capacity of roasted
715 peanut, hazelnut, and almond skins. J. Agric. Food Chem. 57, 10590–10599.
716 https://doi.org/10.1021/jf901391a
717 Mouradov, A., Spangenberg, G., 2014. Flavonoids: a metabolic network mediating plants adaptation to
718 their real estate. Front. Plant Sci. https://doi.org/10.3389/fpls.2014.00620
719 Munin, A. and Edwards-Lévy, F., 2011. Encapsulation of natural polyphenolic compounds; a review.
720 Pharmaceutics, 3(4). doi: 10.3390/pharmaceutics3040793
721 Pasqualone, A., Laddomada, B., Spina, A., Todaro, A., Guzmàn, C., Summo, C., Mita, G., Giannone, V.,
722 2018. Almond by-products: Extraction and characterization of phenolic compounds and evaluation
723 of their potential use in composite dough with wheat flour. LWT - Food Sci. Technol.

https://doi.org/10.1016/j.lwt.2017.10.066

Prgomet, I., Gonçalves, B., Domínguez-Perles, R., Pascual-Seva, N., Barros, A.I.R.N.A., 2017. Valorization challenges to almond residues: Phytochemical composition and functional application. Molecules 22. https://doi.org/10.3390/molecules22101774

Sang, S., Lapsley, K., Jeong, W.S., Lachance, P.A., Ho, C.T., Rosen, R.T., 2002a. Antioxidative phenolic compounds isolated from almond skins (*Prunus amygdalus* Batsch). J. Agric. Food Chem. 50, 2459–2463. https://doi.org/10.1021/jf011533+

Sang, S., Lapsley, K., Rosen, R.T., Ho, C.T., 2002b. New prenylated benzoic acid and other constituents from almond hulls (*Prunus amygdalus* Batsch). J. Agric. Food Chem. 50, 607–609. https://doi.org/10.1021/jf0110194

Sfahlan, A.J., Mahmoodzadeh, A., Hasanzadeh, A., Heidari, R., Jamei, R., 2009. Antioxidants and antiradicals in almond hull and shell (*Amygdalus communis* L.) as a function of genotype. Food Chem. 115, 529–533. https://doi.org/10.1016/j.foodchem.2008.12.049

Smeriglio, A., Mandalari, G., Bisignano, C., Filocamo, A., Barreca, D., Bellocco, E., Trombetta, D., 2016. Polyphenolic content and biological properties of Avola almond (*Prunus dulcis* Mill. D.A. Webb) skin and its industrial byproducts. Ind. Crops Prod. 83, 283–293. https://doi.org/10.1016/j.indcrop.2015.11.089

Takeoka, G.R., Dao, L.T., 2003. Antioxidant constituents of almond [*Prunus dulcis* (Mill.) D.A. Webb] hulls. J. Agric. Food Chem. 51, 496–501. https://doi.org/10.1021/jf020660i

743 **TABLES**

Table 1. Weather conditions (seasons 2015 and 2016) for the almond tree crop under Continental climate in Portugal.

Parameter	Year	Month											
		January	February	March	April	May	June	July	August	September	October	November	December
Air temperature (ºC)	2015	6.67[z]	6.02	10.64	13.28	17.49	21.92	24.65	22.09	18.24	14.12	9.80	7.87
	2016	8.02	7.42	8.52	10.71	14.35	20.11	25.22	24.91	21.38	16.26	9.49	6.33
Precipitation (mm)	2015	13.80	16.80	12.60	43.80	49.80	72.40	11.60	8.00	48.80	122.60	77.60	50.40
	2016	175.00	83.00	47.20	156.00	138.20	15.60	3.20	22.00	31.40	41.60	71.80	35.60
Relative humidity (%)	2015	79.40	71.70	59.00	63.40	55.10	51.20	46.40	50.80	55.90	74.90	87.60	86.50
	2016	85.10	75.90	68.10	70.10	68.30	57.20	44.90	44.30	53.90	70.80	78.10	86.70

[z] Average value of the month

Table 2. Quantification of different phenolic compounds in almond hulls.

Compound (µg/g DW)	Irrigation regime				
	No irrigation	35%	70%	100%	100%-35%
2015					
Phenolic acids					
Protocatechuic acid	2.22 [y] ±0.03 e	2.82±0.05 b	2.51±0.04 d	3.15±0.07 a	2.69±0.07 c
Chlorogenic acid	9.69±0.26 b	11.55±0.52 a	5.63±0.06 d	5.27±0.16 d	6.36±0.13 c
trans-p-coumaric acid	2.49±0.08 c	3.34±0.15 a	2.77±0.10 b	3.38±0.05 a	2.61±0.15 bc
Flavonoids					
Naringenin-7-*O*-glucoside	33.25±0.30 c	104.21±1.28 ab	105.50±0.91 a	103.54±0.82 b	105.56±0.90 a
Kampferol-3-*O*-rutinoside	0.58±0.05 a	0.52±0.04 ab	0.51±0.02 b	0.48±0.04 b	0.37±0.03 c
Kampferol-3-*O*-glucoside	0.42±0.04 c	0.47±0.01 b	0.72±0.04 a	0.49±0.04 b	0.34±0.01 d
Isorhamnetin-3-*O*-rutinoside	2.26±0.14 c	2.53±0.08 b	3.14±0.17 a	2.04±0.05 d	1.84±0.02 e
Isorhamnetin-3-*O*-glucoside	0.56±0.02 a	0.50±0.03 b	0.37±0.00 c	0.55±0.02 ab	0.52±0.05 ab
Isorhamnetin	0.88±0.03 a	0.89±0.01 a	0.48±0.01 d	0.72±0.01 c	0.78±0.05 b
2016					
Phenolic acids					
Protocatechuic acid	2.87±0.07 b	3.63±0.01 a	3.39±0.08 a	3.66±0.07 a	3.59±0.42 a
p-hydroxycinnamic acid	5.67±0.12 a	3.99±0.04 c	2.12±0.14 d	4.71±0.07 b	4.38±0.46 bc
Vanillic acid	1.70±0.02 b	1.04±0.02 c	0.49±0.11 d	2.32±0.02 a	0.99±0.66 cd
Chlorogenic acid	19.62±0.15 a	4.80±0.03 e	5.84±0.24 d	6.21±0.15 c	9.25±0.21 b
trans-p-coumaric acid	2.40±0.04 d	2.67±0.05 c	4.32±0.22 a	2.46±0.07 d	3.66±0.02 b
Flavonoids					
Naringenin-7-*O*-glucoside	19.62±0.15 c	33.53±0.29 b	33.75±0.10 ab	34.16±0.38 a	33.43±0.45 b
Kampferol-3-*O*-rutinoside	1.54±0.03 b	0.87±0.06 e	0.95±0.03 d	1.41±0.07 c	1.73±0.03 a
Kampferol-3-*O*-glucoside	0.89±0.03 c	1.19±0.01 a	0.96±0.04 bc	1.02±0.02 b	1.14±0.12 a
Isorhamnetin-3-*O*-rutinoside	6.11±0.20 d	6.49±0.11 bc	6.30±0.20 cd	7.96±0.30 a	6.79±0.15 b
Isorhamnetin-3-*O*-glucoside	0.92±0.03 a	0.53±0.02 d	0.53±0.02 d	0.76±0.03 b	0.64±0.02 c
Isorhamnetin	1.31±0.07 a	0.76±0.03 c	0.61±0.04 d	1.00±0.05 b	0.71±0.03 c

Means ± SD (n=3) in the same row followed by different lowercase letter are significantly different at $p<0.05$ according to the analysis of variance (ANOVA) and multiple range test (Tukey's test).

744

Table 3. Quantification of different phenolic compounds in almond skins.

Compound (µg/g DW)	Irrigation regime				
	No irrigation	35%	70%	100%	100%-35%
2015					
Phenolic acids					
Protocatechuic acid	0.75 [y] ±0.01 d	1.10±0.00 b	0.91±0.02 c	1.33±0.04 a	1.36±0.05 a
p-hydroxybenzoic acid	1.31±0.02 e	2.99±0.03 a	1.71±0.02 d	2.68±0.08 b	2.56±0.11 c
trans-p-coumaric acid	0.38±0.01 d	0.84±0.01 c	0.83±0.03 c	1.34±0.02 a	1.11±0.03 b
Flavonoids					
Naringenin-7-*O*-glucoside	4.76±0.07 c	9.89±0.10 b	10.01±0.09 b	9.92±0.11 b	10.95±1.01 a
Eriodictyol-7-O-glucoside	1.37±0.07 bc	2.09±0.21 a	1.92±0.33 a	1.05±0.05 c	1.41±0.14 b
(-)-Epicatechin	2.94±0.22 c	4.13±0.09 ab	3.83±0.23 b	4.28±0.37 a	4.38±0.15 a
Kampferol-3-*O*-glucoside	1.39±0.01 b	1.95±0.06 a	1.43±0.08 b	1.89±0.02 a	1.96±0.13 a
Isorhamnetin-3-*O*-rutinoside	5.48±0.04 c	7.10±0.08 a	6.06±0.15 b	7.01±0.20 a	6.21±0.14 b
Isorhamnetin-3-*O*-glucoside	1.60±0.02 c	1.68±0.01 b	1.55±0.05 c	1.90±0.03 a	1.44±0.07 d
Isorhamnetin	1.40±0.04 c	1.93±0.03 a	1.70±0.01 b	1.73±0.01 b	1.69±0.07 b
2016					
Phenolic acids					
Protocatechuic acid	1.28±0.01 d	1.47±0.03 b	1.19±0.02 e	1.37±0.01 c	1.61±0.04 a
p-hydroxybenzoic acid	1.78±0.04 c	1.87±0.02 b	1.83±0.02 bc	1.98±0.05 a	1.55±0.03 d
trans-p-coumaric acid	0.31±0.00 e	0.59±0.03 a	0.42±0.00 d	0.47±0.02 c	0.56±0.01 b
Flavonoids					
Naringenin-7-*O*-glucoside	1.50±0.02 c	4.80±0.01 b	4.75±0.06 b	4.93±0.02 a	1.56±0.03 c
Eriodictyol-7-*O*-glucoside	2.62±0.11 a	1.49±0.06 b	2.64±0.08 a	2.43±0.14 a	2.40±0.30 a
(-)-Epicatechin	4.06±0.11 a	2.49±0.27 c	2.93±0.19 b	2.94±0.33 b	4.44±0.03 a
Kampferol-3-*O*-glucoside	1.70±0.01 a	1.39±0.00 d	1.35±0.00 e	1.41±0.01 c	1.43±0.01 b
Isorhamnetin-3-*O*-rutinoside	6.23±0.06 a	5.52±0.03 bc	5.58±0.02 b	5.51±0.07 bc	5.43±0.08 c
Isorhamnetin-3-*O*-glucoside	1.21±0.07 a	1.03±0.01 bc	0.97±0.01 c	0.87±0.02 d	1.03±0.01 b
Isorhamnetin	2.32±0.02 a	1.74±0.01 d	1.98±0.03 b	2.03±0.03 b	1.83±0.01 c

Means ± SD (n=3) in the same row followed by different lowercase letter are significantly different at $p<0.05$ according to the analysis of variance (ANOVA) and multiple range test (Tukey's test).

745
746
747
748

Table 4. Quantification of different phenolic compounds in blanching water

Compound	Irrigation regime				
(µg/L)	No irrigation	35%	70%	100%	100%-35%
Phenolic acids					
Protocatechuic acid	28.31 ±0.07 a	15.71±0.28 d	19.27±0.43 b	17.57±0.24 c	15.89±0.18 d
p-hydroxybenzoic acid	6.34±1.51 a	5.05±0.07 ab	3.56±0.04 b	3.90±0.09 b	6.49±1.87 a
Chlorogenic acid	28.44±0.57 a	18.05±0.12 d	27.62±0.58 ab	25.87±0.42 b	21.23±2.75 c
Flavonoids					
Eriodictyol-7-*O*-glucoside	32.40±4.72 a	24.32±0.13 c	34.35±0.92 a	30.59±0.57 ab	26.28±4.47 bc
Kampferol-3-*O*-glucoside	16.77±2.13 b	20.68±0.91 a	15.72±0.49 b	17.04±0.25 b	19.39±1.10 a
Isorhamnetin-3-*O*-rutinoside	61.59±4.01 c	80.31±0.88 a	60.53±1.08 c	65.82±0.88 b	80.33±1.61 a

Means ± SD (n=3) in the same row followed by different lowercase letter are significantly different at $p<0.05$ according to the analysis of variance (ANOVA) and multiple range test (Tukey's test).

749

Table 5. Antibacterial activity of almond by-products.

Antibiotics (% RIZD)	Samples	Standard bacterial strains					
		L. monocytogenes ATCC	*S. aureus* MJS241	*E. faecalis* MJS257	*P. aeruginosa* ATCC	*E. coli* MJS260	*K. pneumoniae* MJS281
Gentamycin 10 ug disc^{-1}	Hulls 2015	49 b	¤ □	¤ □	>100 a	0	0
	Hulls 2016	44 b	¤ □	¤ □	97 a	0	0
	Skins 2015	47 b	¤ □	¤ □	97 a	0	0
	Skins 2016	56 a	¤ □	¤ □	>100 a	0	0
p-value		*			*n.s.*		
Gentamycin 30 ug disc-^{1}	Hulls 2015	45 a	¤ □	¤ □	81 a	0	0
	Hulls 2016	40 a	¤ □	¤ □	76 a	0	0
	Skins 2015	44 a	¤ □	¤ □	76 a	0	0
	Skins 2016	52 a	¤ □	¤ □	92 a	0	0
p-value		*n.s.*			*n.s.*		
Ciprofloxacin 10 ug disc^{1}	Hulls 2015	45 ab	¤ □	¤ □	50 b	0	0
	Hulls 2016	40 b	¤ □	¤ □	47 b	0	0
	Skins 2015	44 b	¤ □	¤ □	47 b	0	0
	Skins 2016	52 a	¤ □	¤ □	57 a	0	0
p-value		*			*		

Means ± SD (n=3) in the same column followed by different lowercase letter are significantly different at $p<0.05$ according to the analysis of variance (ANOVA) and multiple range test (Tukey's test); * $p<0.05$, n.s. not significant
0 – without effect;
0-100 – less effective than an antibiotic;
> 100 – more effective than an antibiotic;
¤ □- extract effective and antibiotic without effect.

750

751

Table 6. Pearson correlation of individual almond by-products phenolics with antioxidant capacity

	2015			2016		
	ABTS	DPPH	FRAP	ABTS	DPPH	FRAP
			Hulls			
Chlorogenic acid	0.528 *	0.532 *	0.710 **	0.941 ***	0.839 ***	0.928 ***
Isorhamnetin-3-*O*-glucoside	0.572 *	0.644 **	0.524 *	0.647 **	0.559 *	0.682 **
Isorhamnetin	0.684 **	0.770 ***	0.839 ***	0.683 **	0.664 **	0.739 **
			Skins			
Eridictyol-7-*O*-glucose	0.626 *	0.604 *	0.605 *	0.589 *	0.536 *	0.515 *
Isorhamnetin-3-*O*-rutinose	0.581 *	0.514 *	0.530 *	0.559 *	0.502 *	0.616 **
Isorhamnetin	0.683 **	0.751 **	0.796 ***	0.757 **	0.730 **	0.704 **

* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

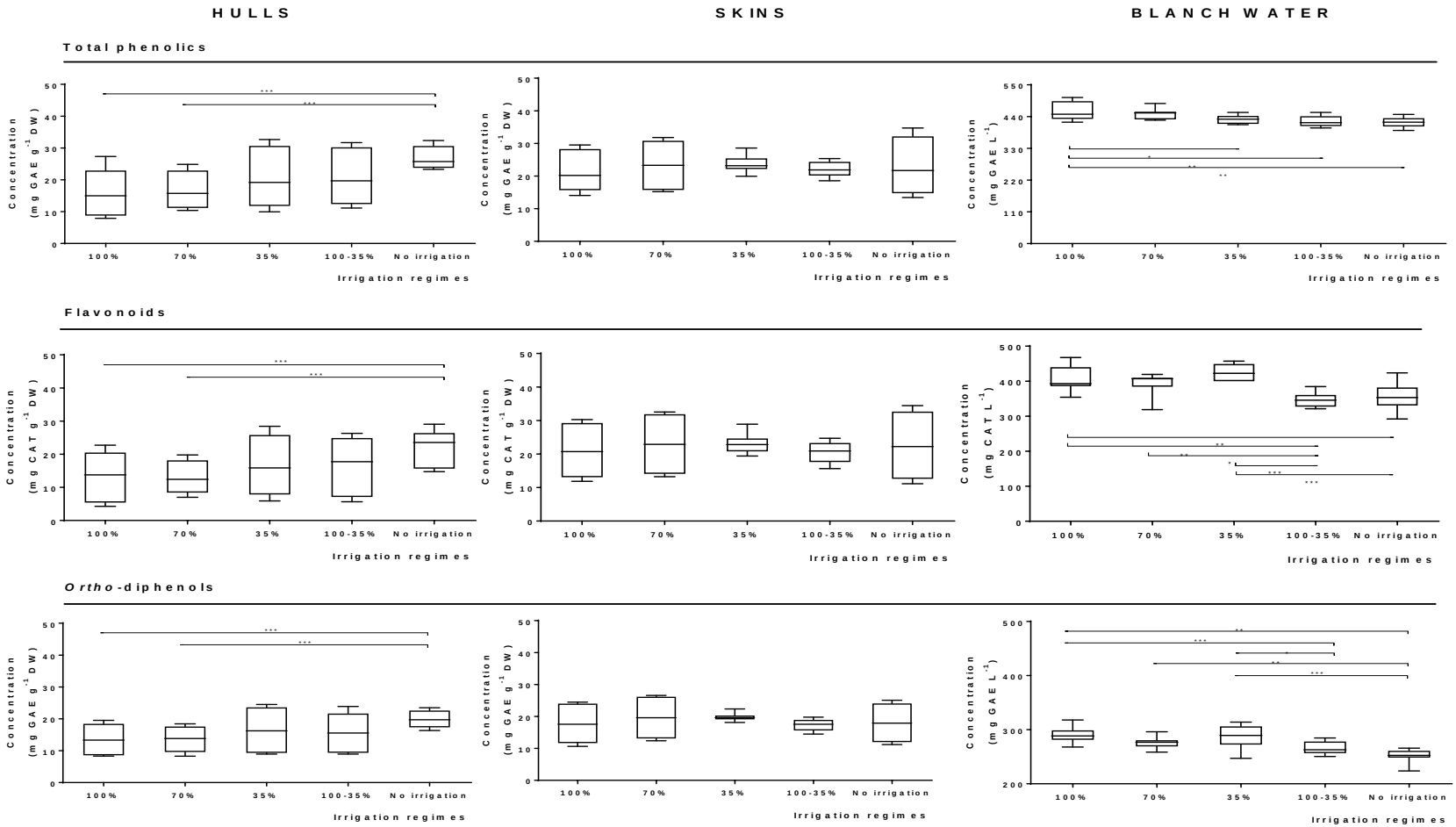


Figure 1. Box plots with quartiles (upper values 75.0%, median 50.0%, and lower values 25.0%) of total phenolics, *ortho*-diphenols, and flavonoids of hydromethanolic extracts of almond by-products and blanching water. "Δ" indicates outlier data. Statistical differences were according to the analysis of variance and the multiple range test (Tukeys' test). Statistical differences were set at $p<0.05$(*), $p<0.01$(**), and $p<0.001$(***).

Figure 2.

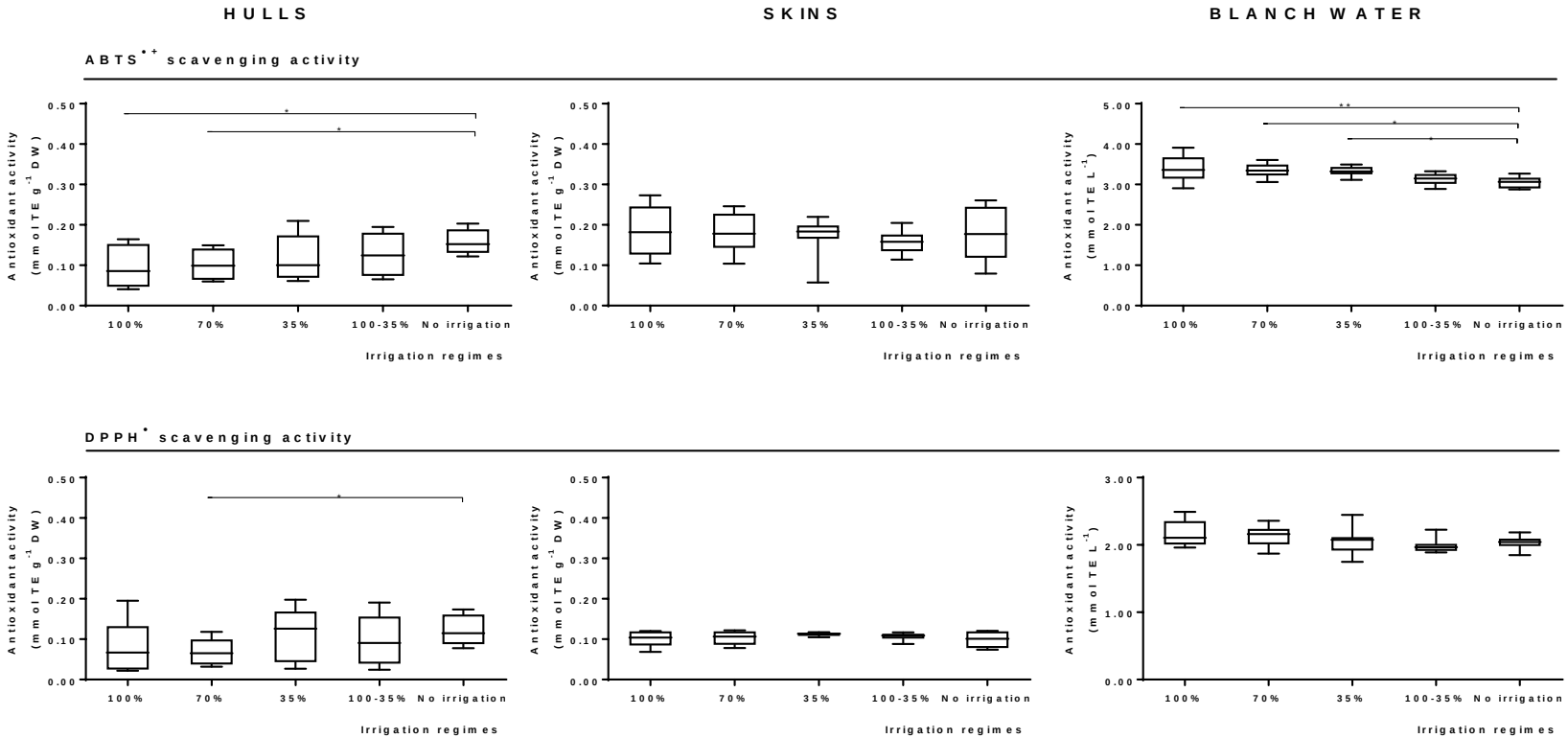
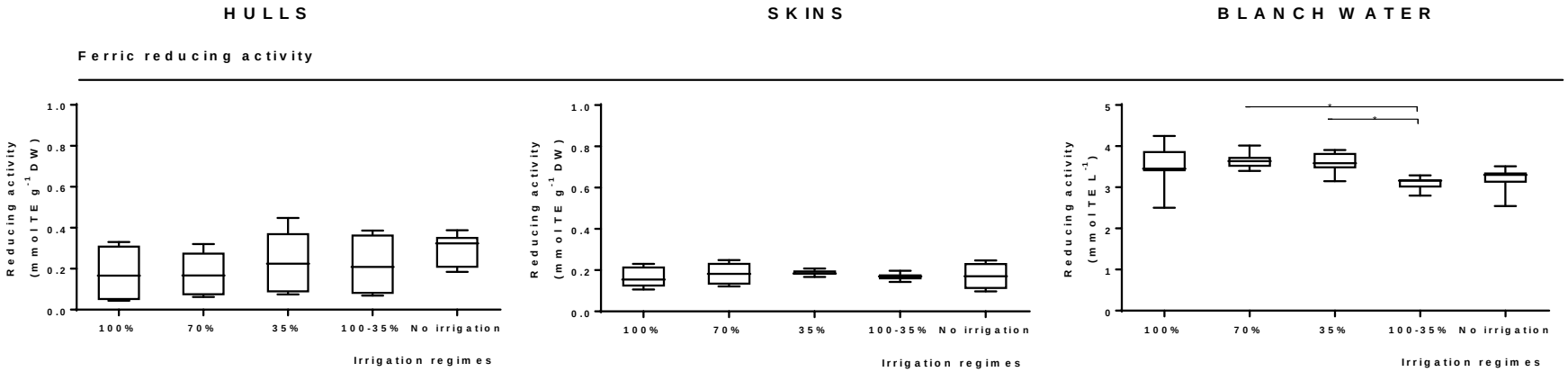


Figure 2. Box plots with quartiles (upper values 75.0%, median 50.0%, and lower values 25.0%) of DPPH• and ABTS•+ scavenging activity of hydromethanolic extracts of almond by-products and blanching water. "Δ" indicates outlier data. Statistical differences were according to the analysis of variance and the multiple range test (Tukeys' test). Statistical differences were set at $p<0.05$(*) and $p<0.01$(**).

762

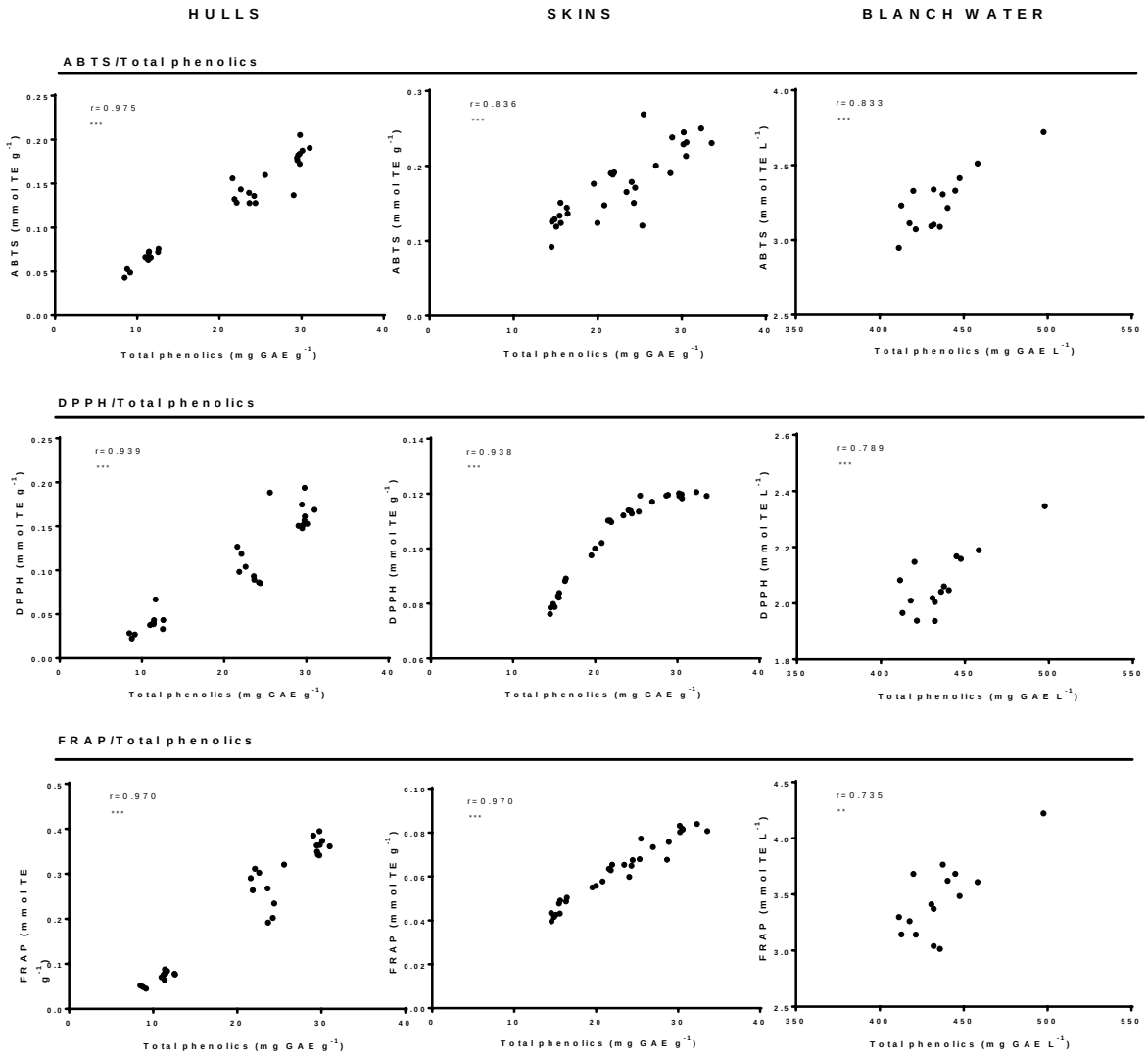
763 **Figure 3.**



764

765 **Figure 3**. Box plots with quartiles (upper values 75.0%, median 50.0%, and lower values 25.0%) of ferric reducing antioxidant activity (FRAP) of hydromethanolic extracts
766 of almond by-products and blanching water. "Δ" indicates outlayer data. Statistical differences were according to the analysis of variance and the multiple range test (Tukeys'
767 test). Statistical differences were set at $p<0.05$(*).

768 **Figure 4.**



769
770
771 **Figure 4.** Pearson correlation of total phenols with DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activity, and ferric
772 reducing antioxidant activity (FRAP) of hydromethanolic extracts of almond by-products and blanching
773 water. Statistical differences were according to the analysis of variance and the multiple range test
774 (Tukeys' test). Statistical differences were set at p<0.01(**) and p<0.001(***).
775