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Study of Zalema Grape Pomace: Phenolic Composition and Biological Effects in *Caenorhabditis elegans*

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ABSTRACT: The phenolic composition of the extractable fraction of Zalema grape pomace has been analyzed by HPLC-DAD-MS and consisted of mainly flavanols and flavonols (122.75 and 23.11 mg/100 g dry pomace, respectively). The antioxidant activity has been determined by in vitro FRAP, ABTS, and ORAC assays (11.7, 34.9, and 63.6 mmol of Trolox equivalents (TE) per 100 g of dry pomace, respectively) and in vivo using the model organism *Caenorhabditis elegans*. Cultivation of *C. elegans* in media containing 100 μg/mL dry pomace extract increased the survival of worms submitted to thermally induced oxidative stress, whereas a decrease in the rate of worm survival was found for 300 μg/mL extract. Interestingly, the levels of reactive oxygen species (ROS) were significantly decreased in stressed worms treated with the pomace extract at the two concentration levels. Further studies are required to explain this unexpected behavior, as well as to determine the compounds and mechanisms involved in the observed effects.

KEYWORDS: *phenolic compounds, byproducts, grape pomace, Zalema, antioxidant activity, C. elegans, oxidative stress*

INTRODUCTION

Winemaking generates a variety of residues consisting of seeds, skins, and stems that cause environmental and economical problems, which could be minimized by the exploitation and valorization of those products. Grape pomace is recognized as an important source of phenolic compounds (flavonoids and nonflavonoids). The main subclasses of phenolic compounds in white grape pomaces are flavanols, flavonols, and phenolic acids. These compounds have received attention because of their antioxidant,[1] anti-inflammatory,[2] and antimicrobial activities[3] and have been related with the prevention of important chronic pathologies such as cardiovascular disorders,[4] neurodegenerative decline,[5] or cancer.[6]

Oxidative stress is related to the physiopathology of many diseases[7−9] and takes place when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system, which can generate important cell damage.[10] ROS include superoxide anion radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^{\bullet}$). These species can be generated by endogenous (incomplete reduction of oxygen at the respiratory chain in the mitochondria) or exogenous sources (drugs, metal ions, heat, ionizing radiation, UV light, pathogens, inflammatory cytokines). Although ROS excess is toxic, ROS are necessary as cell signaling molecules and can also mediate the adaptive stress response of cells.[11] Beneficial effects of polyphenols on health have been associated with their ability to decrease ROS accumulation, thus reducing cell damage.[12,13] Nevertheless, although polyphenols are usually recognized as antioxidants and free radical scavengers, it is also well-known that they are able to act as pro-oxidants in in vivo situations,[14] depending on the type of compounds, their concentration, and the biological system. However, whereas high levels of pro-oxidant activity are expected to produce toxic effects, light pro-oxidant effects might also be beneficial, because by imposing a mild degree of oxidative stress, as might be produced by diet polyphenols, the levels of antioxidant defenses and xenobiotic-metabolizing enzymes might be raised, leading to overall cytoprotection.[11,14]

Several in vitro methods had been employed to measure the antioxidant activity of polyphenols extracted from different plant sources including grapes and byproducts, such as oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP).[15]

Caenorhabditis elegans is a nematode that has been used as a model organism in in vivo studies of antioxidant activity, especially related to stress resistance, aging, and degenerative diseases.[13,16−19] It possesses a short lifespan (around 20 days at 22 °C) and is easy to culture and to manipulate in the laboratory. Furthermore, its multicellularity, with a complete system of tissues and organs, raises the possibility to consider metabolism of compounds. There is also correlation in cellular and molecular principles between *C. elegans* and mammals, with 60−80% of the human gene homologues being identified in *C.*

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elegans.[20] For these reasons, it is a good model to study biological effects of beneficial and toxic substances as well as to identify new pharmacological targets. Studies with apple,[21] blueberry,[22] onion,[23] tea,[24,25] spinach,[26] and *Ginkgo biloba*[18,27] extracts rich in polyphenols have been carried out in *C. elegans* for evaluation of their biological effects. As far as we know, no previous studies regarding grape pomace extracts have been published in this respect.

In this work the biofunctional potential of Zalema grape pomace, a white grape variety grown exclusively in southwestern Spain, has been evaluated. Its phenolic composition was analyzed, and its antioxidant activity has been tested in in vitro assays (ABTS, FRAP, and ORAC) and in the model organism *C. elegans* regarding resistance to stress and ROS accumulation.

MATERIALS AND METHODS

Standards and Reagents. Gallic acid, (+)-catechin (C), (−)-epicatechin (EC), quercetin, quercetin 3-*O*-rutinoside (rutin), sodium carbonate, potassium persulfate, fluorescein (FL), 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), ampicillin sodium salt, nistatine, agar, yeast extract, fluorodeoxyuridine (FUdR), phosphate-buffered saline (PBS), and cholesterol were purchased from Sigma-Aldrich (Madrid, Spain). Isorhamnetin, quercetin 3-*O*-glucoside, and kaempferol 3-*O*-glucoside were obtained from Extrasynthese (Barcelona, Spain). Procyanidin dimers B1, B2, B3, and B4 and trimer C1 were isolated in the laboratory by semipreparative HPLC.[28] 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka (Madrid, Spain), and HPLC grade acetonitrile was from Carlo Erba (Rodano, Italy). Analytical grade glacial acetic acid, glycine, Folin reagent, methanol, formic acid, and iron trichloride ($FeCl_3{\cdot}6H_2O$) were obtained from Panreac (Barcelona, Spain), and dimethyl sulfoxide (DMSO) was from Scharlau Chemie (Barcelona, Spain).

Samples. Grape pomace of the variety Zalema, D.O. "Condado de Huelva" (Spain), from the 2011 harvest collected after winemaking was supplied by Vinícola del Condado winery (Bollullos Par del Condado, Spain) and further freeze-dried.

The dry pomace was extracted with 75% methanol according to the methodology described by Gonzalez-Manzano et al.,[29] with some modifications. The sample (50 g) was homogenized in 250 mL of the solvent, kept under shaking for 1 h in a shaking apparatus (VWR Incubating minishaker), and further centrifuged at 4190*g* for 15 min; the supernatant was collected and the residue submitted to the same process twice. The supernatants were combined, methanol was eliminated under reduced pressure, and the aqueous extract esd washed with *n*-hexane. Afterward, it was passed through a C18 column (10 × 5 cm) first eluted with water and then with methanol. The obtained methanolic extract was concentrated to dryness and freeze-dried.

HPLC-DAD-ESI/MS Analysis. Analyses were carried out in a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C18, 3 μm (4.6 × 150 mm), column thermostated at 35 °C was used. The solvents used were (A) 0.1% formic acid and (B) acetonitrile. The elution gradient established was 0% B to 15% B in 35 min and from 15 to 40% B over 10 min, with re-equilibration of the column using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD at 280 and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple-quadrupole ion trap mass analyzer, which was controlled by Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and as turbo gas (400 °C) for solvent drying (40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupoles were set at unit resolution. The ion spray voltage was operated at −4500 V in the negative mode. Method settings were as follows: declustering potential (DP), −40 V; entrance potential (EP), −10 V; collision energy (CE), −50 V; and cell exit potential (CXP) −3 V. To obtain the fragmentation pattern of the parent ion, enhanced product ion (EPI) mode was also applied using the following settings: declustering potential (DP), −50 V; entrance potential (EP), −6 V; collision energy (CE), −25 V; and collision energy spread (CES) 0 V.

Phenolic compounds were identified by their retention time, UV−vis spectra, and mass spectra and by comparison with our data library and standards when available. The compounds were quantified from the areas of their chromatographic peaks recorded at 280 and 370 nm, for flavanols and flavonols, respectively.

Calibration curves were constructed for the following polyphenols: catechin, epicatechin, procyanidin dimers B1, B2, B3, and trimer C1, quercetin-3-*O*-rutinoside (rutin), quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, and isorhamnetin. Epicatechin 3-*O*-gallate, procyanidin B2 3-*O*-gallate, galloyled procyanidin dimers, and gallocatechin−catechin (GC−C) dimers were quantified with the calibration curve of procyanidin B2 and trimers and tetramers using the curve of trimer C1. Quercetin 3-*O*-glucuronide, quercetin 3-*O*-galactoside, and quercetin *O*-pentose were quantified as quercetin 3-*O*-glucoside, kaempferol-3-*O*-glucuronide was quantified as kaempferol-3-*O*-glucoside, and isorhamnetin 3-*O*-glucoside was quantified as isorhamnetin.

The samples were analyzed in triplicate, and the results were expressed as milligrams polyphenol per 100 g of dry pomace.

FRAP Assay. Ferric reducing ability was evaluated according to Benzie and Strain[30] with some modifications. The FRAP reagent contained 10 mM TPTZ solution in 40 mM HCl, 20 mM $FeCl_3{\cdot}6H_2O$, and acetate buffer (300 mM, pH 3.6) (1:1:10, v/v/v). The extract (100 μL, containing 5 mg of dry material) was added to 3 mL of the FRAP reagent, and the absorbance was measured at 593 nm after incubation at room temperature for 6 min, using the FRAP reagent as a blank. Different dilutions of the extract were assayed, and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30−1000 μM). Three independent experiments in triplicate were performed for each of the assayed extracts, and the results were expressed as Trolox-equivalent antioxidant capacity (TEAC), here considered as the millimoles of Trolox with the same antioxidant capacity as 100 g of the studied extract.

ABTS/Persulfate Assay. The $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulfate (2.45 mM) in water.[31] The mixture was allowed to stand in the dark at room temperature for 16 h before use, and then the $ABTS^{\bullet+}$ solution was diluted with phosphate-buffered saline (PBS) at pH 7.4 to give an absorbance of 0.7 ± 0.02 at 734 nm. The extract (50 μL, containing 2.5 mg of dry material) was mixed with 2 mL of the $ABTS^{\bullet+}$ diluted solution and vortexed for 10 s, and the absorbance was measured at 734 nm after 4 min of reaction at 30 ∘C.

Different dilutions of the extract were assayed, and the results were obtained by interpolating the absorbance on a calibration curve obtained with Trolox (30−1000 μM). Three independent experiments in triplicate were performed for each of the assayed extracts, and the results were expressed as Trolox-equivalent antioxidant capacity (TEAC; mmol of Trolox with the same antioxidant capacity as 100 g of the studied extract).

ORAC Assay. The ORAC assay was carried out following a method reported previously.[32] A Synergy HT Multi-Mode microplate reader (Biotek, Winooski, VT, USA) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm was used. The measurements were made in plates with 96 white flat-bottom wells (Biomol). The reference standard used was a 20 μM Trolox solution that was prepared in PBS. A FL stock solution (100 μmol/L) in PBS (75 mmol/L, pH 7.4) was prepared and kept at 4 °C in the dark. Fresh working FL solution (100 nmol/L) was prepared by diluting the stock solution with the PBS.

In each well, 50 μL of FL (78 nM) and 50 μL of extract (containing 2.5 mg of dry material), blank (PBS), or standard (20 μM Trolox

solution) were placed. The plate was heated to 37 °C for 15 min, and then 25 μL of AAPH (221 mM) was added. The fluorescence was measured and measurements were then taken every 5 min until the reading had decreased to <5% of the initial reading. Three independent experiments in triplicate were performed for each of the assayed extracts, and the results were expressed as Trolox-equivalent antioxidant capacity (TEAC; mmol of Trolox with the same antioxidant capacity as 100 g of the studied extract).

Assays with *C. elegans*. *Strains and Maintenance Conditions.* *C. elegans* strains wild type N2 were obtained from the *Caenorhabditis* Genetics Centre at the University Minnesota (Minneapolis, USA). All strains were routinely propagated at 20 °C on nematode growth medium (NGM) plates with heat killed (30 min at 65 °C) *Escherichia coli* strain OP50 as a food source. Synchronization of worm cultures was achieved by treating gravid hermaphrodites with bleach (12% aqueous solution of 10%, *w/v*, sodium hypochlorite). The suspension was shaken vigorously during one min and kept a further min in ice; this process was repeated five times. Eggs were resistant to bleach whereas worms were dissolved in the bleach solution. The suspension was centrifuged (2 min, 2000*g*) and the complete process repeated twice. The pellet containing the eggs was washed four times with an equal volume of buffer M9 (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1 M $MgSO_4$, H_2O to 1 L). The supernatant was removed and the eggs resuspended and kept in a small volume of M9. Around 100 to 200 μL of the M9 with eggs (depending on eggs concentration) were transferred and incubated in NGM agar plates. The dry pomace extract dissolved in DMSO was added to the nematode growth medium during its preparation to get final concentrations in plates of 100 and 300 μg/mL. Quercetin (60 μg/mL) dissolved in DMSO was used as positive control. Control plates were prepared containing the same volume of DMSO (0.1% DMSO, v/v).

Stress Assays. Oxidative stress in worms was induced by submitting the animals to a temperature of 35 °C that provokes damage caused by accumulation of ROS.[33] L1 larvae were transferred to NGM agar plates (⌀ 100 mm) containing the pomace extract (100 and 300 μg/mL) and cultivated at 20 °C; simultaneous assays were also performed on control plates without pomace extract. When the worms reached the L4 stage (2 days), they were transferred to new plates with and without pomace extract but also containing FUdR at a concentration of 150 μM to prevent reproduction and progeny overgrowth. At the second day of adulthood the worms were transferred again to fresh plates also containing FUdR and the different treatments until they reached the fourth day of adulthood, when they were transferred with a platinum wire to agar plates (⌀ 35 mm, 20 worms per plate) lacking pomace extract, and switched to 35 °C for 8 h in an incubator. After that time, dead and alive nematodes were counted. The total time of exposure of the worms (from L1 to fourth day of adulthood) to the pomace extract before they were submitted to the thermal stress was 6 days. Assays were performed with approximately 100 nematodes per treatment. Experiments were performed in triplicate for each of the assayed extracts and quercetin. The relative rates of survival of worms after being submitted to thermal stress were expressed in relation to the untreated controls.

Accumulation of Reactive Oxygen Species (ROS). The accumulation of ROS was evaluated at the end of the 6 day cultivation period (4th day of adulthood) with and without the pomace extract. Cellular ROS were quantified by the dichlorofluorescein assay using a microplate reader.[34] Briefly, the worms were individually transferred to the well of a 96-well plate containing 75 μL of PBS and then exposed to thermal stress (2 h at 35 °C), after which 25 μL of DFCH-DA solution in PBS buffer was added to each well (final concentration of DFCH in the well was 62.5 μM). The acetate groups of DFCH-DA were removed in worm cells, and the released DFCH is oxidized by intracellular ROS to yield the fluorescent dye DCF. The fluorescence from each well was measured at 35 °C immediately after incorporation of the reagent and every 10 min for 60 min with a 1 s integration time, using 485 and 535 nm as excitation and emission wavelengths, respectively. Recording of the DCF fluorescence intensity with time in single worms was used as an index of the individual intracellular levels of ROS. The response of the method was checked every day using a H_2O_2 curve. Three independent experiments were performed per treatment, and for each experiment ROS measurements were made in at least 24 individual worms. The measurements were performed in an Ultra Evolution Multi-functional microplate reader (Tecan, NC, USA).

Statistical Analysis. The statistical analyses were performed using Statistica v 8.0 software (StatSoft Inc., 2007). ANOVA was applied to make the multiple comparisons of values to determine possible significant differences between treated and control groups in oxidative stress and ROS assays. Significant difference was statistically considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of Phenolic Compounds in Zalema Grape Pomace Extract. Figure 1 shows chromatograms recorded at 280 and 370 nm with the phenolic profile of the grape pomace extract of the Zalema variety. Seventeen flavanols, nine flavonols, one hydrolyzable tannin, and one hydroxybenzoic acid could be assigned and quantified (Table 1). Flavanols were the major components with a total concentration around 111 mg/100 g dry pomace. Different procyanidins with low degree of polymerization (dimer to tetramer) could be separated and quantified. The most

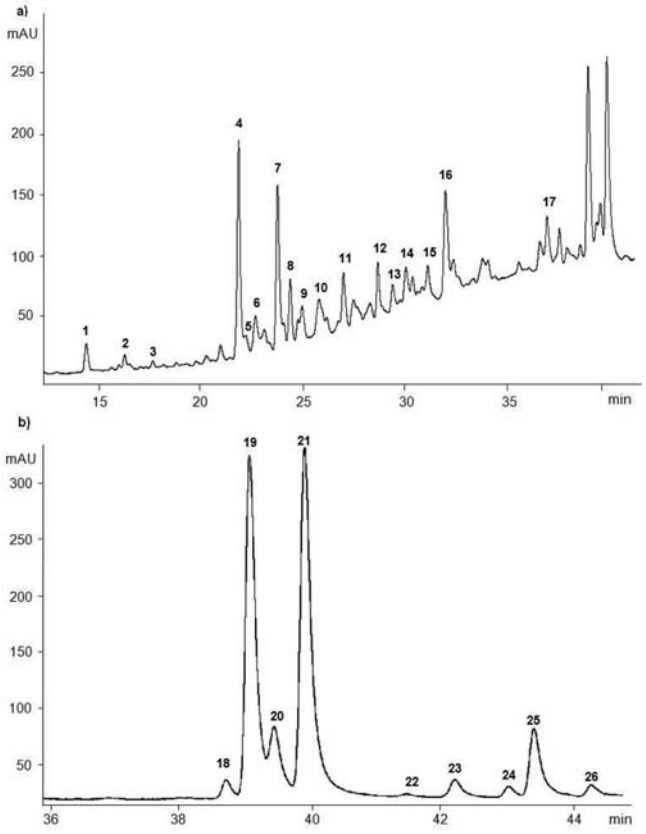

Figure 1. (a) HPLC chromatograms of pomace extract at 280 nm. Peaks: 1, trimer C2; 2, GC−C dimer 1; 3, GC−C dimer 2; 4, procyanidin B1; 5, tetramer 1; 6, procyaninin B3; 7, catechin; 8, C−C−EC trimer; 9, tetramer 2; 10, procyanidin B4; 11, procyanidin B2; 12, epicatechin; 13, galloyled procyanidin 1; 14, galloyled procyanidin 2; 15, trimer C1; 16, procyanidin B2 3-*O*-gallate; 17, epicatechin gallate. (b) HPLC chromatograms of pomace extract at 370 nm. Peaks: 18, quercetin 3-*O*-rutinoside; 19, quercetin 3-*O*-glucuronide; 20, quercetin 3-*O*-galactoside; 21, quercetin 3-*O*-glucoside; 22, quercetin pentose; 23, kaempferol 3-*O*-glucoside 1; 24, kaempferol 3-*O*-glucuronide; 25, kaempferol 3-*O*-glucoside 2; 26, isorhamnetin 3-*O*-glucoside.

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Table 1. Concentration of Phenolic Compounds Identified in Grape Pomace of the Zalema Variety, Retention Times, and Mass Spectrometric Data in the HPLC-DAD-MS Analysis

phenolic compound	retention time (min)	MS (m/z)[a] $[M - H]^-$	MS/MS (m/z)[a]	concentration (mg/100 g)[b]
Flavanols				
catechin (C)	23.62	289	245	14.66 ± 0.07
epicatechin (EC)	28.54	289	245	5.79 ± 0.08
epicatechin gallate	36.85	441	289, 169	5.39 ± 0.05
procyanidin B1	21.71	577	425, 405, 289	22.40 ± 0.03
procyanidin B2	26.85	577	425, 405, 289	5.76 ± 0.02
procyanidin B3	22.54	577	425, 405, 289	5.41 ± 0.07
procyanidin B4	25.66	577	425, 405, 289	7.15 ± 0.14
procyanidin B2 3-*O*-gallate	31.86	729	577, 425, 407, 289	13.94 ± 0.42
galloyled procyanidin 1	29.28	729	577, 425, 407, 289	3.47 ± 0.17
galloyled procyanidin 2	29.92	729	577, 425, 407, 289	4.31 ± 0.05
GC−C dimer 1	16.11	593	425, 407, 325	1.57 ± 0.09
GC−C dimer 2	17.49	593	425, 407, 325	1.11 ± 0.01
trimer C2	14.23	865	577, 289	3.66 ± 0.01
C−C−EC trimer	24.24	865	577, 289	7.38 ± 0.08
trimer C1	30.99	865	577, 289	2.87 ± 0.45
tetramer 1	22.05	1153	863, 577, 287	1.78 ± 0.14
tetramer 2	24.82	1153	863, 577, 287	4.50 ± 0.14
Flavonols				
quercetin 3-*O*-rutinoside	38.48	609	301	1.74 ± 0.25
quercetin 3-*O*-glucuronide	38.871	477	301	9.41 ± 0.06
quercetin 3-*O*-galactoside	39.28	463	301	2.14 ± 0.04
quercetin 3-*O*-glucoside	39.79	463	301	11.15 ± 0.06
quercetin pentose	41.48	433	301	0.24 ± 0.06
kaempferol hexoside	42.31	447	285	0.65 ± 0.09
kaempferol 3-*O*-glucuronide	43.22	461	285	1.06 ± 0.04
kaempferol 3-*O*-glucoside	43.64	447	285	1.56 ± 0.02
isorhamnetin 3-*O*-glucoside	44.6	477	315	0.94 ± 0.03
Phenolic Acids				
gallic acid	11.63	169	125	tr[c]
Hydrolyzable Tannins				
monogalloyl glucose	15.52	331	271, 169	tr

[a]Fragment ion detected in negative ion MS/MS. [b]Each value represents mean (n = 9) ± SD: mg polyphenol/100 g dry grape pomace. [c]tr, traces.

abundant flavanol was procyanidin B1 followed by catechin, procyanidin B2 3-*O*-gallate, C−C−EC trimer, and procyanidin B4. Flavanols are known to be prominent compounds in grape byproducts such as grape seeds and pomace.[35,36] These compounds are widely recognized to possess antioxidant activity demonstrated in in vitro and in vivo studies.[37,38]

Another subclass of flavonoids detected and quantified in the grape pomace extract was flavonols, which represent 21% of the total flavonoids of the extract (28.89 mg/100 g). The main flavonols were quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide, in agreement with the results reported by Kammerer et al.[34] in white grape seeds and skins. Other flavonols detected were different *O*-glycosides from the aglycones quercetin and kaempferol (Table 1). Other detected phenolic compounds in the pomace extract were gallic acid and a gallotannin, tentatively identified as monogalloylglucose according to its mass spectral characteristics. In a previous paper by our group,[39] gallic acid and other phenolic acids were found to be relevant compounds in Zalema white wines (up to ≈20 mg/L). However, in the Zalema pomace extract only gallic acid was detected in nonquantifiable levels. The presence of gallotannins is not very common in *Vitis vinifera* grapes, but these compounds have been described in seeds and skin of muscadine grape (i.e., *Vitis rotundifolia*).[40]

In Vitro Antioxidant Evaluation of Zalema Grape Pomace Extract. The in vitro antioxidant activity of the Zalema grape pomace extract was assessed using FRAP, ABTS, and ORAC assays. The FRAP assay evaluates the ability of a substance to reduce Fe^{3+} to Fe^{2+}, therefore determining the reducing capacity of a substance.[30] The ABTS assay measures the ability of a compound to scavenge the $ABTS^{\bullet+}$ radical cation.[41] In this study, the ABTS assay was performed at a pH value of 7.4, close to physiological conditions, using persulfate for $ABTS^{\bullet+}$ generation. The ORAC is a standardized method based on the inhibition of free radical-induced oxidation of azo-derived compounds such as AAPH and measurement of the fluorescence of fluorescein, which is subjected to the action of the free radical generator.[32] Table 2 shows the results obtained.

Table 2. Antioxidant Activity in Extract of Grape Pomace of the Zalema Variety

method	value[a]
FRAP	11.7 ± 0.56
ABTS	34.9 ± 0.61
ORAC	63.6 ± 1.81

[a]Each value represents mean (n = 9) ± SD. TEAC value: mmol TE/100 g dry pomace.

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TEAC values (mmol of Trolox showing the same antioxidant capacity as 100 g of the studied extract) were 11.7, 34.9, and 63.6 for FRAP, ABTS, and ORAC methods, respectively. Other authors have also reported relevant antioxidant activity for extracts obtained from grape and byproduct[42,43] greater than those of synthetic food antioxidants BHA, BHT, and ascorbyl palmitate or natural food antioxidants such as vitamin E.[44−46]

The antioxidant capacity of the pomace extract should be attributed to its phenolic content, particularly to flavanols (catechins and proanthocyanidins) and flavonols. These compounds are well represented in the Zalema pomace extract and have been related with the antioxidant activity of plant extracts. The flavonol quercetin has been reported as better antioxidant than catechins, as it possesses higher electron-donating activity[47] and better ability for electron delocalization and stabilization of the produced phenoxyl radicals.[47,48] At a given pH value, quercetin is able to generate a greater proportion of phenolate ions involved in electron transfer than catechins, due to the greater acidity of its phenolic hydroxyls, although this ability is reduced with the substitution of the hydroxyl groups,[46] as occurs in the pomace extract where quercetin is under glycosylated forms.

Assays in *C. elegans*. To check if the pomace polyphenol-rich extract increased the resistance of *C. elegans* against oxidative damage, the worms were submitted to a thermal stress, which is associated with damage caused by accumulation of ROS.[33] The stress (35 °C, 8 h) was applied at the fourth day of adulthood after being grown in the presence of two concentrations of the pomace extract (100 and 300 μg/mL) and the positive control (quercetin 60 μg/mL) in the culture media, and the results were compared with those obtained in control worms grown in the absence of polyphenols.

The assayed concentrations of polyphenols (100 and 300 μg/mL) in the culture medium could be associated with those present in some food sources, such as cocoa, black currant, tea, or apples.[49−52] It is, however, assumed that the level of polyphenols uptake by *C. elegans* is rather low, especially in the case of catechins, as concluded in previous studies by our group.[16,17] As can be observed in Figure 2, the survival rate significantly increased in thermally stressed worms treated with 100 μg/mL of pomace extract (129% of survival relative to untreated worms, $p < 0.05$) and quercetin (130%, $p < 0.05$), but it decreased at 300 μg/mL (54%, $p < 0.05$). This suggests that pomace polyphenols induced a protection of the worms against oxidative stress only at low concentrations, whereas high levels would have detrimental effects. Quercetin, used as positive control, has been reported[16] as a compound that increases the resistance of *C. elegans* against thermal stress. In this study, the results showed similar rates of survival between quercetin and extract at 100 μg/mL ($p > 0.05$) (Figure 2). Different authors have reported improvements in the resistance against thermal and chemically induced oxidative stress in *C. elegans* after treatment with polyphenol-rich extracts, such as *Ginkgo biloba*[27] (100 μg/mL) and cocoa extracts[53] (4 mg/mL). Furthermore, different pure flavonoids, such as catechins,[17,24,25] and flavonols, such as quercetin, kaempferol, fisetin, and rutin,[13,17,18] assayed at different concentrations have been shown to decrease oxidative damage in *C. elegans* submitted to thermal stress. Nevertheless, toxic effects of polyphenols have also been reported in worms submitted to stress conditions and exposed to high concentrations of tannic acid and ellagic acid (from 510 to 680 μg/mL and from 90 to 120 μg/mL, respectively) in the culture media.[54] The authors concluded that elevated concentrations of polyphenols could shorten the lifespan and also increase stress sensitivity.

Figure 3a shows the results on ROS accumulation expressed as percentage of fluorescence relative to controls (worms not

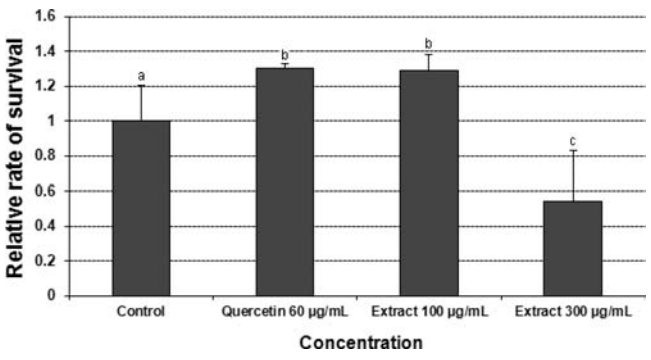

Figure 2. Thermal stress resistances in worms cultivated with pomace extract (100 and 300 μg/mL) and quercetin (60 μg/mL). The results are expressed as the percentage of surviving worms when they were submitted to thermal stress (35 °C at 8 h) compared to control. Statistical significance of differences between control and treated worms was determined by ANOVA (different letters between bars indicate significant difference, $p < 0.05$).

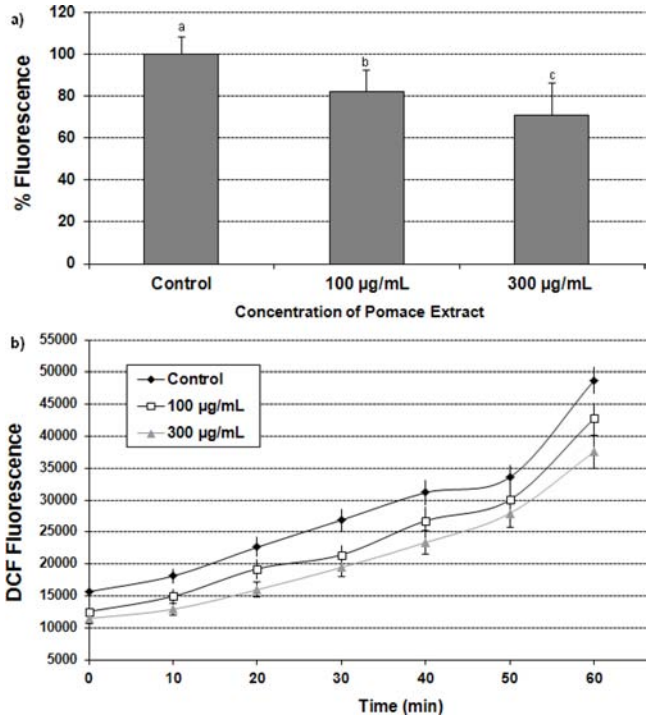

Figure 3. Accumulation ROS was evaluated at the fourth day of adulthood in worms cultured with and without the pomace extract (100 and 300 μg/mL) and exposed to thermal stress (2 h at 35 °C). The fluorescence from each well was measured at 35 °C immediately after incorporation of dichlorofluorescein and every 10 min for 60 min. (a) ROS accumulation at 10 min. Results are expressed as percentage of fluorescence in relation to control animals. (b) ROS accumulation during 60 min. ROS production is expressed as fluorescence units measured after incorporation of the reagent (0 min). Statistical significance of differences between control and treated worms was determined by ANOVA (different letters between bars indicate significant difference, $p < 0.05$).

exposed to the pomace extract) and measured 10 min after the fluorescent probe was added. It could be observed that the worms treated with the pomace extract at both 100 and 300 μg/mL showed significantly ($p < 0.05$) lower ROS levels than controls (82 and 71% of fluorescence relative to control, respectively). Similar differences were maintained over a time of measurement of 60 min (Figure 3b).

The protective effects against thermal and oxidative stress provided by flavonoids in *C. elegans* have been suggested to be due to their ability to decrease intracellular ROS accumulation,[13,22,55] which is in agreement with the result observed in the assays performed in the presence of 100 μg/mL pomace extract. However, there would be a strong disagreement with the results obtained at 300 μg/mL, a concentration found to decrease the resistance to thermal stress (Figure 2). Indeed, certain cellular ROS levels are necessary, as they mediate the adaptive stress response of cells[11] and may be used by the immune system to fight against foreign agents.[56] Thus, an excessive ROS decrease might lead the cell to be less prepared against oxidative insult. Further studies are, however, necessary to determine if the decreased survival observed in worms submitted to the higher concentration of pomace extract (300 mg/L) might be related with the decreased level of ROS.

Winemaking leads to the generation of large quantities of grape wastes that can be used for the extraction of polyphenols. This study has shown that Zalema pomace could be considered a good source of antioxidant polyphenols with potential interest in pharmaceutical and food industries to be incorporated in dietary supplements or as antioxidants in food.

The preliminary results obtained in assays with the model organism *C. elegans* suggest that polyphenol-rich pomace extracts could attenuate ROS accumulation and increase the resistance against oxidative stress when used at relatively low concentrations, although they may be detrimental at higher levels. Further studies are now required to elucidate the reasons, compounds, and mechanisms involved in these effects.

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Notes

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ABBREVIATIONS USED

ABTS, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; AAPH, 2,2′-azobis(2-amidinopropane)-dihydrochloride; C, catechin; *C. elegans*, *Caenorhabditis elegans*; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; EC, epicatechin; FL, fluorescein; FRAP, ferric reducing antioxidant power; FUdR, fluorodeoxyuridine; GC−C, gallocatechin−catechin; HPLC-DAD-ESI/MS, high-performance liquid chromatography−diode array detection−electrospray ionization/mass spectrometry; NGM, nematode growth medium; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TE, Trolox equivalent; TEAC, Trolox-equivalent antioxidant capacity; TPTZ, 2,4,6-tris(2-pyridyl)-*s*-triazine

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