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 analysed 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 the *in vitro* FRAP, ABTS and ORAC assays (11.7, 34.9 and 63.6 mmols of trolox equivalents (TE) per 100 g of dry pomace, respectively), and in *in vivo* using the model organism *Caenorhabditis elegans*. Cultivation of *C. elegans* in media containing 100 µg/mL of 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 of 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 try and explain this unexpected behaviour, as well as to determine the compounds and mechanisms involved in the observed effects.

Keywords

phenolic compounds, by-products, 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 valorisation of those products. Grape pomace is recognised as an important source of phenolic compounds (flavonoids and non-flavonoids). 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 defence 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•). 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, they are also necessary as cell signalling molecules and can also mediate the adaptive stress response of cells[11]. Beneficial effects of polyphenols on health have been associated to 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, since by imposing a mild degree of oxidative stress, as might be produced by diet polyphenols, the levels of antioxidant defences and xenobiotic-metabolising 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 by-products, such as the oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picryhidrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the 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, ageing and degenerative diseases[13,16-19]. It possesses a short lifespan (around 20 days at 22 ºC) 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 genes homologues being identified in *C. 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 the Zalema grape pomace, a white grape variety grown exclusively in southwest 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

Standars and reagents

Gallic acid, (+)-catechin (C), (-)-epicatechin (EC), quercetin, quercetin 3-*O*-rutinoside (rutin), sodium carbonate, potassium persulphate, 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 semi-preparative HPLC[28]. ABTS (2,2-azino-bis-(3-ethylbenzothiazolne-6-sulfonic acid) diammonium salt) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-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.

131 The dry pomace was extracted with 75% methanol according to the methodology
132 described by Gonzalez-Manzano et al.[29], with some modifications. The sample (50 g)
133 was homogenized in 250 mL of the solvent, kept under shaking for 1 h in a shaking
134 apparatus (VWR Incubating minishaker) and further centrifuged at 4,190 *g* for 15 min;
135 the supernatant was collected and the residue submitted to the same process twice. The
136 supernatants were combined, methanol was eliminated under reduced pressure and the
137 aqueous extract washed with n-hexane. Afterwards it was passed through a C18 column
138 (10 x 5 cm) first eluted with water and then with methanol. The obtained methanolic
139 extract was concentrated to dryness and freeze-dried.
140
141 **HPLC-DAD-ESI/MS analysis**
142 Analyses were carried out in a Hewlett-Packard 1100 chromatograph (Agilent
143 Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector
144 (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters
145 Spherisorb S3 ODS-2 C18, 3 μm (4.6 x 150 mm) column thermostatted at 35 °C was
146 used. The solvents used were: (A) 0.1% formic acid, and (B) acetonitrile. The elution
147 gradient established was 0% B to 15% B in 35 min, and from 15-40% B over 10 min,
148 and re-equilibration of the column using a flow rate of 0.5 mL/min. Double online
149 detection was carried out in the DAD at 280 and 370 nm as preferred wavelengths and
150 in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.
151 MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt,
152 Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer,
153 which was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer
154 gas (30 psi) and as turbo gas (400 ºC) for solvent drying (40 psi). Nitrogen served as the
155 curtain (20 psi) and collision gas (medium). Both quadrupols were set at unit resolution.

The ion spray voltage was operated at -4500V in the negative mode. Method settings were: declustering potential (DP), -40 V; entrance potential (EP), -10 V; collision energy (CE), -50V; and cell exit potential (CXP) -3 V. In order 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), -25V; and collision energy spread (CES) 0 V.

Phenolic compounds were identified by their retention time, UV-vis spectra and mass spectra, and comparison with our data library and standards when available. The compounds were quantified from the areas of their chromatographic peaks recorded at 280 nm 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, kaempherol-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 as kaempferol-3-*O*-glucoside, and isorhamnetin 3-*O*-glucoside as isorhamnetin.

The samples were analyzed in triplicate and the results expressed as mg polyphenol/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 of 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 (30-1,000 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 mmols of Trolox with the same antioxidant capacity as 100 g of the studied extract.

ABTS/persulphate assay

The $ABTS^{\bullet+}$ radical was produced by the oxidation of 7 mM ABTS with potassium persulphate (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, vortexed for 10 s, and the absorbance 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 (30–1,000 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; mmols of Trolox with the same antioxidant capacity as 100 g of the studied extract).

206 **ORAC assay**

207 The ORAC assay was carried out following a method reported previously[32]. A Synergy
208 HT Multi-Mode Microplate Reader (Biotek® Winooski, EEUU) with fluorescence
209 filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm
210 was used. The measurements were made in plates with 96 white flat-bottom wells
211 (Biomol®). The reference standard used was a 20 μM Trolox solution that was prepared
212 in PBS. A FL stock solution (100 μmol/L) in PBS (75 mmol/L, pH 7.4) was prepared
213 and kept at 4 ºC in the dark. Fresh working FL solution (100 nmol/L) was prepared by
214 diluting the stock solution with the PBS.
215 In each well, 50 μL of FL (78 nM) and 50 μL of extract (containing 2.5 mg of dry
216 material), blank (PBS), or standard (20 μM Trolox solution) were placed. The plate was
217 heated to 37 ºC for 15 min and then 25 μL of AAPH (221 mM) were added. The
218 fluorescence was measured and measurements were then taken every 5 min until the
219 reading had decreased to less than 5% of the initial reading. Three independent
220 experiments in triplicate were performed for each of the assayed extracts and the results
221 were expressed as Trolox-equivalent antioxidant capacity (TEAC; mmols of Trolox
222 with the same antioxidant capacity as 100 g of the studied extract).
223
224 **Assays with *C. elegans***
225 **Strains and maintenance conditions**
226 *C. elegans* strains wild type N2 were obtained from the *Caenorhabditis* Genetics Centre
227 at the University Minnesota (Minneapolis, USA). All strains were routinely propagated
228 at 20 ºC on nematode growth medium (NGM) plates with heat killed (30 min at 65 ºC)
229 *Escherichia coli* strain OP50 as a food source. Synchronization of worm cultures was
230 achieved by treating gravid hermaphrodites with bleach (12% aqueous solution of 10%,

231 *w/v*, sodium hypochlorite). The suspension was shaken vigorously during one min and
232 kept a further min in ice; this process was repeated five times. Eggs were resistant to
233 bleach whereas worms were dissolved in the bleach solution. The suspension was
234 centrifuged (2 min, 2000 *g*) and the complete process repeated twice. The pellet
235 containing the eggs was washed four times with an equal volume of buffer M9 (3 g
236 KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1M $MgSO_4$, H_2O to 1 L). The supernatant was
237 removed and the eggs resuspended and kept in a small volume of M9. Around 100 to
238 200 μL of the M9 with eggs (depending on eggs concentration) were transferred and
239 incubated in NGM agar plates. The dry pomace extract dissolved in DMSO was added
240 to the nematode growth medium during its preparation to get final concentrations in
241 plates of 100 and 300 μg/mL. Quercetin (60 μg/mL) dissolved in DMSO was used as
242 positive control. Control plates were prepared containing the same volume of DMSO
243 (0.1% DMSO, *v/v*).
244
245 **Stress assays**
246 Oxidative stress in worms was induced submitting the animals at a temperature of 35 ºC
247 that provokes damage caused by accumulation of ROS[33]. L1 larvae were transferred to
248 NGM agar plates (∅ 100 mm) containing the pomace extract (100 and 300 μg/mL) and
249 cultivated at 20 ºC; simultaneous assays were also performed on control plates without
250 pomace extract. When the worms reached the L4 stage (2 days), they were transferred to
251 new plates with and without pomace extract but also containing FUdR at a
252 concentration of 150 μM to prevent reproduction and progeny overgrowth. At the 2nd
253 day of adulthood the worms were transferred again to fresh plates also containing FUdR
254 and the different treatments until they reached the 4th day of adulthood, when they were
255 transferred with a platinum wire to agar plates (∅ 35 mm, 20 worms per plate) lacking

of pomace extract, and switched to 35 ºC 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 4th day of adulthood) to the pomace extract before submitting them 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 reactive oxygen species (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 ºC) after which 25 µL of DFCH-DA solution in PBS buffer was added to each well (final concentration of DFCH in the well 62.5 µM). The acetate groups of DFCH-DA are removed in worm cells and the released DFCH is oxidised by intracellular ROS to yield the fluorescent dye DCF. The fluorescence from each well was measured at 35 ºC immediately after incorporation of the reagent and every 10 min for 60 min with a 1 s of 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 in order 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 hydrolizable 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 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 by-products as grape seeds and pomace[35,36]. These compounds are widely recognised to possess antioxidant activity demonstrated in *in vitro* and *in vivo* studies[37,38].

Another sub-class of flavonoids detected and quantified in the grape pomace extract were flavonols, which represent 21% of the total flavonoids of the extract (28.89 mg/100g). 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

306 seeds and skins. Other flavonols detected were different *O*-glycosides from the
307 aglycones quercetin and kaempferol (Table 1). Other detected phenolic compounds in
308 the pomace extract were gallic acid and a gallotannin, tentatively identified as
309 monogalloylglucose according to its mass spectral characteristics. In a previous paper of
310 our group[39], gallic acid and other phenolic acids were found to be relevant compounds
311 in Zalema white wines (up to ≈20 mg/L). However, in the Zalema pomace extract only
312 gallic acid was detected in non-quantifiable levels. The presence of gallotannins is not
313 very common in *Vitis vinifera* grapes, but these compounds have been described in
314 seeds and skin of Muscadine grape (*i.e., Vitis rotundifolia*)[40].
315
316 ***In vitro* antioxidant evaluation of Zalema grape pomace extract**
317 The *in vitro* antioxidant activity of the Zalema grape pomace extract was assessed using
318 FRAP, ABTS and ORAC assays. The FRAP assay evaluates the ability of a substance
319 to reduce Fe^{3+} to Fe^{2+}, therefore determining the reducing capacity of a substance[30]. The
320 ABTS assay measures the ability of a compound to scavenge the $ABTS^{\bullet+}$ radical
321 cation[41]. In this study, the ABTS assay was performed at a pH value of 7.4, close to
322 physiological conditions, using persulphate for $ABTS^{\bullet+}$ generation. The ORAC is a
323 standardized method based on the inhibition of free radical-induced oxidation of azo-
324 derived compounds such as AAPH and measurement of the fluorescence of fluorescein
325 which is subjected to the action of the free radical generator[32]. Table 2 shows the results
326 obtained. TEAC values (mmols of Trolox showing the same antioxidant capacity as 100
327 g of the studied extract) were 11.7, 34.9 and 63.6 for FRAP, ABTS and ORAC
328 methods, respectively. Other authors have also reported relevant antioxidant activity for
329 extracts obtained from grape and by-product[42,43], greater than synthetic food
330 antioxidants BHA, BHT, ascorbyl palmitate or natural food antioxidants like vitamin
331 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 delocalisation and stabilisation of the produced phenoxyl radicals[47,48]. At a given pH value, quercetin is able to generate 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*

In order 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 ºC, 8h) was applied at the 4th 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 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 to those present in some food sources, such as cocoa, blackcurrant, 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 of our group[16,17]. As it can be observed in the 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 only induced a protection of the worms against oxidative stress 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 like 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 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 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 both at 100 and 300 μg/mL showed significant ($p<0.05$) lower ROS levels than controls (82% and 71% of fluorescence relative to

381 control, respectively). Similar differences were maintained over a time of measurement
382 of 60 min (Figure 3b).
383 The protective effects against thermal and oxidative stress provided by flavonoids in *C.*
384 *elegans* have been suggested to be due to their ability to decrease intracellular ROS
385 accumulation[13,55,22], which is in agreement with the result observed in the assays
386 performed in the presence of 100 μg/mL of pomace extract. However, there would be a
387 strong disagreement with the results obtained at 300 μg/mL, a concentration found to
388 decrease the resistance to thermal stress (Figure 2). Indeed certain cellular ROS levels
389 are necessary, as they mediate the adaptive stress response of cells[11] and may be used by
390 the immune system to fight against foreign agents[56]. Thus, an excessive ROS decrease
391 might lead the cell to be less prepared against oxidative insult. Further studies are,
392 however, necessary to determine if the decreased survival observed in worms submitted
393 to the higher concentration of pomace extract (300 mg/L) might be related with the
394 decreased level of ROS.
395
396 Winemaking leads to the generation of large quantities of grape wastes that can be used
397 for the extraction of polyphenols. This study has shown that Zalema pomace could be
398 considered a good source of antioxidant polyphenols with potential interest in
399 pharmaceutical and food industries to be incorporated in dietary supplements or as
400 antioxidants in food.
401 The preliminary results obtained in assays with the model organism *C. elegans* suggest
402 that polyphenol-rich pomace extracts could attenuate ROS accumulation and increase
403 the resistance against oxidative stress when used at relatively low concentrations,
404 although they may be detrimental at higher levels. Further studies are now required to
405 elucidate the reasons, compounds and mechanisms involved in these effects.
406

ABBREVIATIONS USED

ABTS	2,2-azino-bis-(3-ethylbenzothiazolne-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-picryhidrazyl
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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673 **Figure captions**

674

675 **Figure 1. a)** HPLC chromatograms of pomace extract at 280 nm: 1, trimer C2; 2, GC-C
676 dimer 1; 3, GC-C dimer 2; 4, procyanidin B1; 5, tetramer 1; 6, procyaninin B3; 7,
677 catechin; 8, C-C-EC trimer; 9, tetramer 2; 10, procyanidin B4; 11, procyanidin B2; 12,
678 epicatechin; 13, galloyled procyanidin 1; 14, galloyled procyanidin 2; 15, trimer C1; 16,
679 procyanidin B2 3-*O*-gallate; 17, epicatechin gallate. **b)** HPLC chromatograms of
680 pomace extract at 370 nm: 18, quercetin 3-*O*-rutinoside; 19, quercetin 3-*O*-glucuronide;
681 20, quercetin 3-*O*-galactoside; 21, quercetin 3-*O*-glucoside; 22, quercetin pentose; 23,
682 kaempferol 3-*O*-glucoside 1; 24, kaempferol 3-*O*-glucuronide; 25, kaempferol 3-*O*-
683 glucoside 2; 26, isorhamnetin 3-*O*-glucoside.

684

685 **Figure 2.** Thermal stress resistances in worms cultivated with pomace extract (100 and
686 300 μg/mL) and quercetin (60 μg/mL). The results are expressed as the percentage of
687 survival worms when were submitted to thermal stress (35 ºC at 8h) compared to
688 control. Statistical significance of differences between control and treated worms were
689 determined by ANOVA (different letters between bars indicate significant difference,
690 $p<0.05$).

691

692 **Figure 3.** The accumulation ROS was evaluated at 4th day of adulthood in worms
693 cultured with and without the pomace extract (100 and 300 μg/mL) and exposed to
694 thermal stress (2 h at 35 ºC). The fluorescence from each well was measured at 35 ºC
695 immediately after incorporation of dichlorofluorescein and every 10 min for 60 min. **a)**
696 ROS accumulation at 10 min. Results are expressed as percentage of fluorescence in
697 relation to control animals **b)** ROS accumulation during 60 min. ROS production is

698 expressed as fluorescence units measured after incorporation of the reagent (0 min).
699 Statistical significance of differences between control and treated worms were
700 determined by ANOVA (different letters between bars indicate significant difference,
701 $p<0.05$).

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/100g)[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	traces
Hydrolizable tannins				
Monogalloyl glucose	15.52	331	271, 169	traces

[a]Fragment ion detected in negative ion MS/MS

[b]mg polyphenol/100 g dry grape pomace. Each value represents mean (n=9) ± SD.

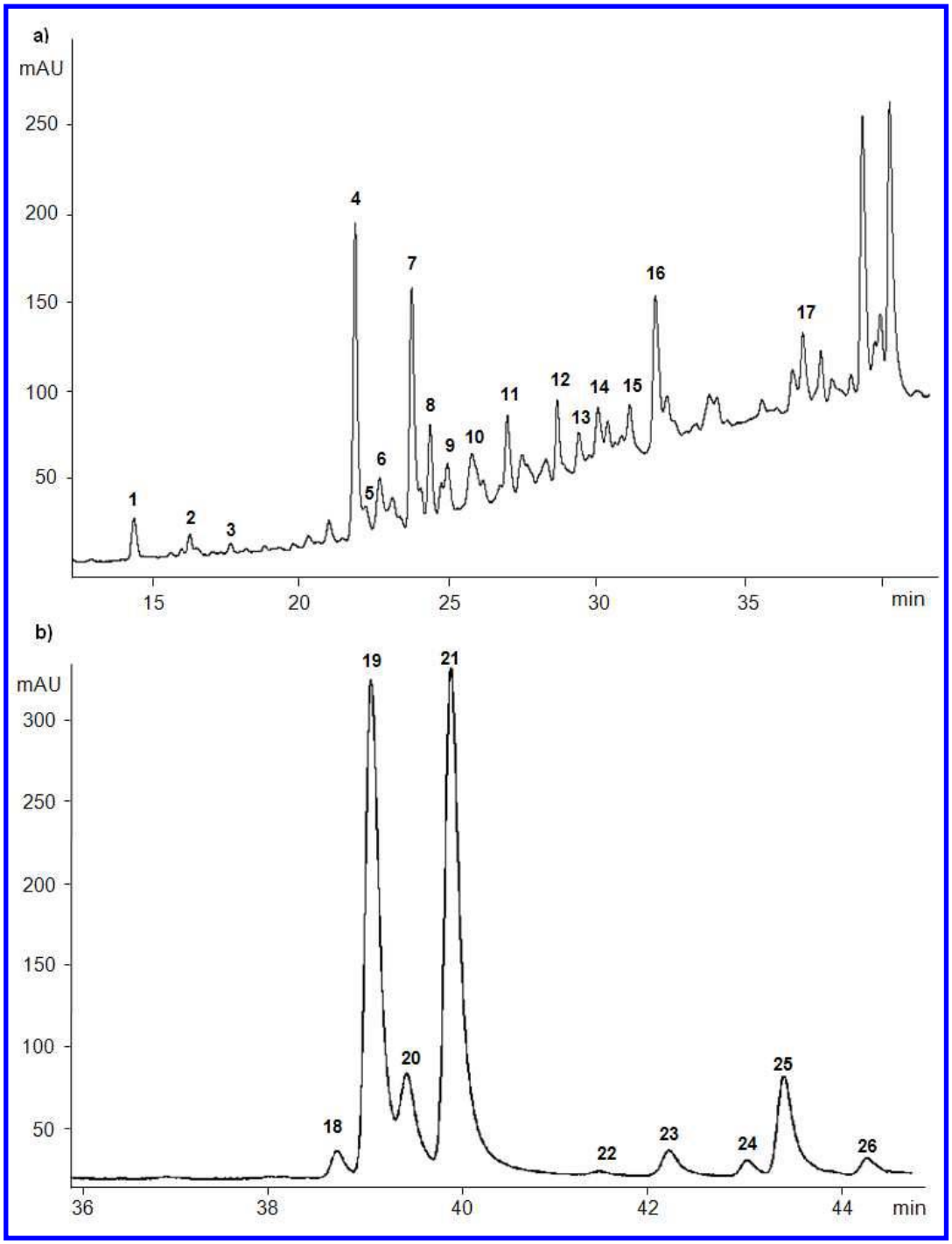
Table 2. Antioxidant Activity in Extract of Grape Pomace of the Zalema Variety.

Method	Value[a]
FRAP	11.7 ± 0.56
ABTS	34.9 ± 0.61
ORAC	63.6 ± 1.81

[a] TEAC value: mmols TE/100 g dry pomace.

Each value represents mean (n=9) ± SD.

Figure 1


a)
mAU
250
200
150
100
50
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
15
20
25
30
35
min
b)
mAU
300
250
200
150
100
50
18
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21
22
23
24
25
26
36
38
40
42
44
min

Figure 2.

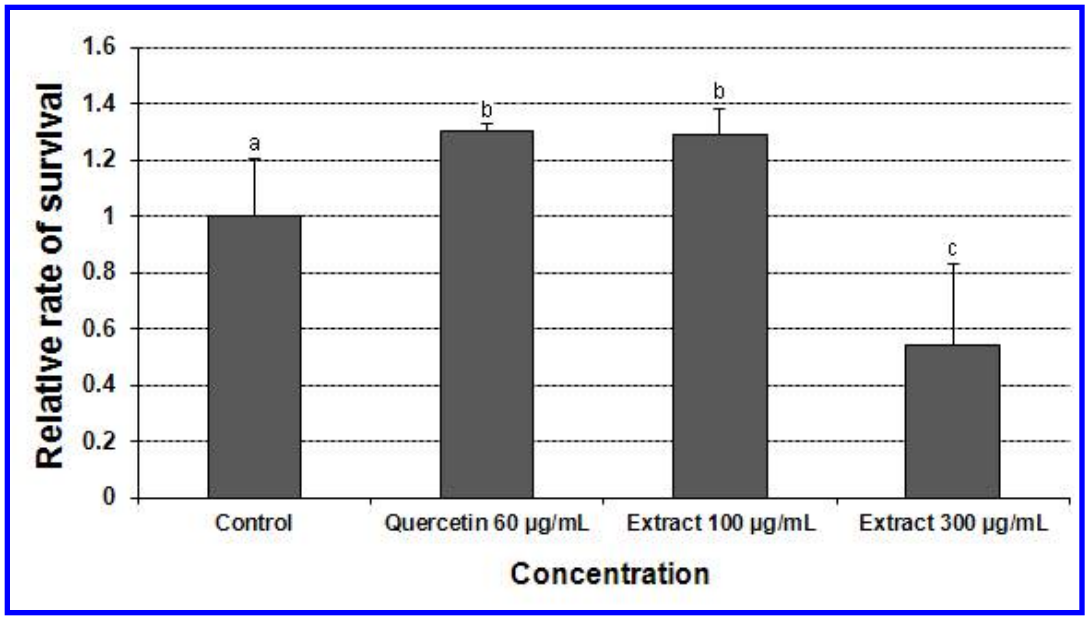
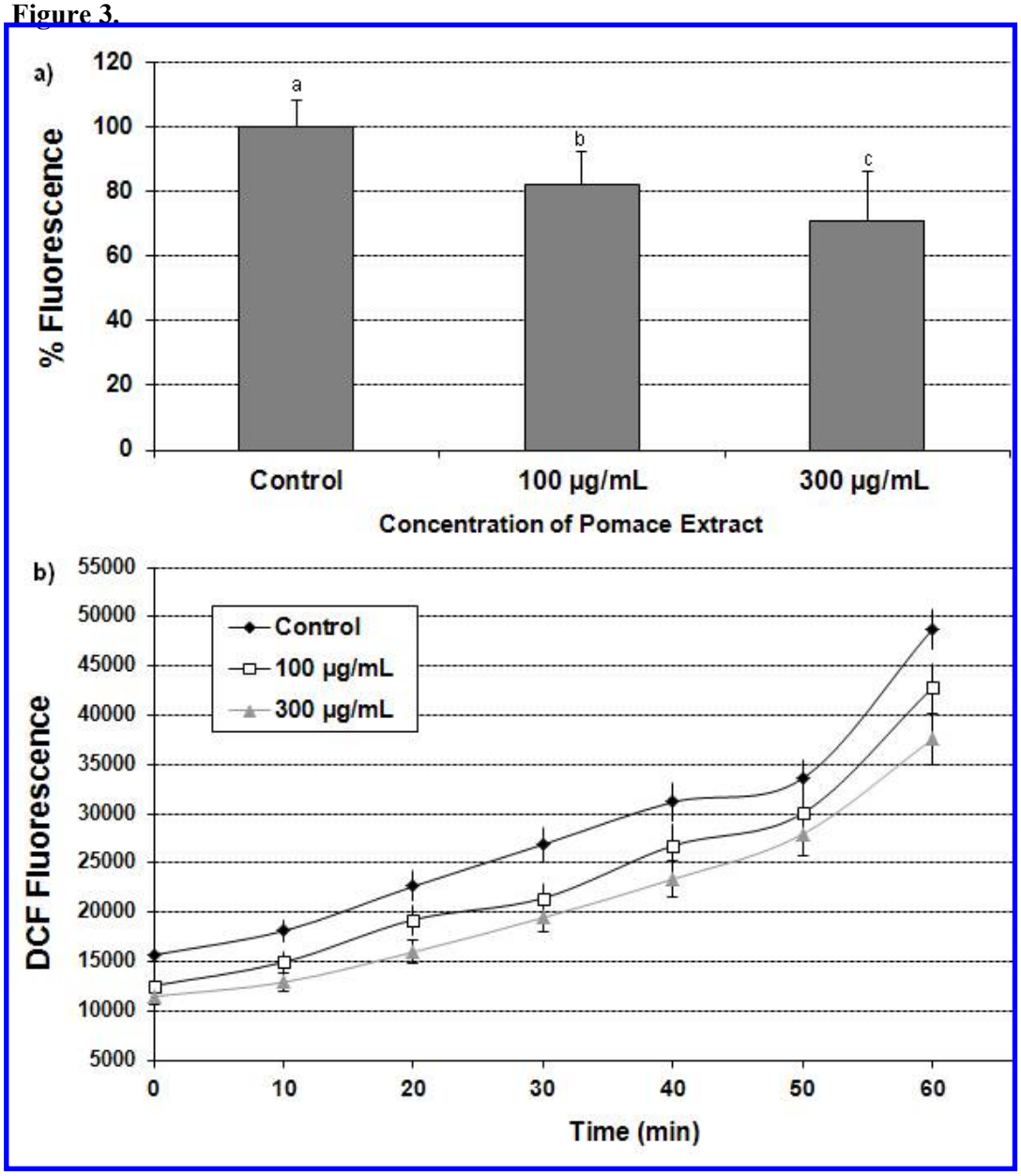
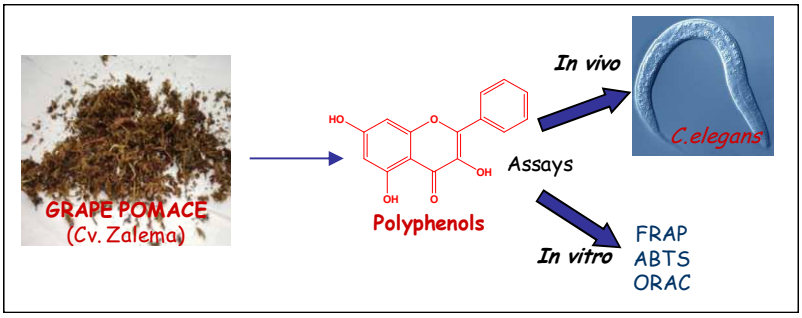

Relative rate of survival
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
b
c
Control
Quercetin 60 μg/mL
Extract 100 μg/mL
Extract 300 μg/mL
Concentration

Figure 3.


a)
% Fluorescence
120
100
80
60
40
20
0
a
b
c
Control
100 µg/mL
300 µg/mL
Concentration of Pomace Extract
b)
DCF Fluorescence
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
Control
100 µg/mL
300 µg/mL
0
10
20
30
40
50
60
Time (min)


GRAPE POMACE
(Cv. Zalema)
HO
O
OH
OH
O
Polyphenols
Assays
In vivo
C.elegans
In vitro
FRAP
ABTS
ORAC