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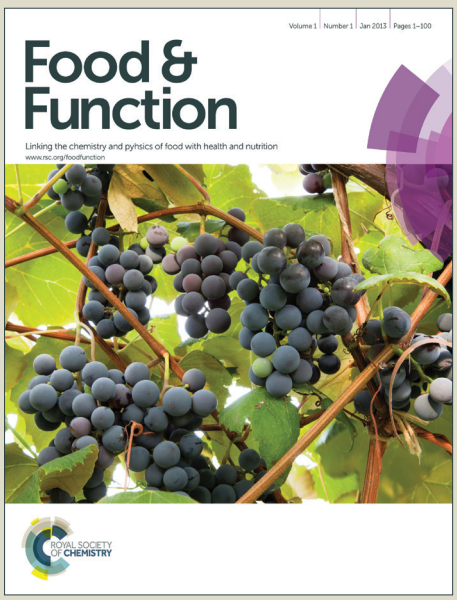

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Supercritical Fluid Extraction of grape seeds: Extract chemical composition, antioxidant activity and inhibition of nitrite production in LPS-stimulated Raw 264.7 cells

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Title Running Head: Biological properties of SFE grape seed fatty acids

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ABSTRACT

Grape by-products are a rich source of bioactive compounds having broad medicinal properties, but usually wasted from juice/wine processing industries. The present study investigates the use of Supercritical Fluid Extraction (SFE) to obtain an extract rich in bioactive compounds. First, some variables involved in the extraction were applied. SFE conditions were selected based on the oil mass yield, fatty acid profile and total phenolic composition. As a result, 40 ºC and 300 bar were selected as operational conditions. The phenolic composition of the grape seeds oil was determined using LC-DAD. The antioxidant activity was determined by ABTS and DPPH assays. For the anti-inflammatory activity the inhibition of nitrite production was assessed. The grape seed oil extracted was rich in phenolic compounds and fatty acids with significant antioxidant and anti-inflammatory activity. From these results, an added economical value to this agroindustrial residue is proposed using techniques environmental friendly.

Keywords: Supercritical Fluid Extraction (SFE), by-products, ABTS, DPPH, anti-inflammatory

Introduction

Liquid and solid wastes produced by food processing industries are increasing nowadays. Disposal of these waste materials containing biodegradable organic matter can create environmental problems. Efficient, inexpensive and environmentally rational utilization of agricultural by-products is of undisputed importance for higher profitability and minimal environmental impact. One of the higher value options is the recovery of bioactive plant food constituents, which could be used in pharmaceutical, cosmetics and food industry.[1]

Grapes (*Vitis vinifera* L.) are widely cultivated at climate zone all over the world. Wine and juice are the main products of this crop generating large quantities of waste including grape skins and seeds. When grape is processed into wine or juice making, some of the bioactive compounds are extracted into the juice; however, still appreciable amount remains in the pomace or the seeds[2] (Beveridge, Girard, Kopp, & Drover, 2005). As a consequence, grape by-products constitute a very cheap source for the extraction of antioxidants with potential health promoting and disease protective qualities.[3,4] Antioxidant extracts can then be used as dietary supplements or in the production of phytochemicals, thus providing important economic advantages.[5,6] Grape seeds contain about 14–17% of oil and consist mainly of triglycerides and triacylglycerols of fatty acid. The main interest in this oil lies in its high contents of unsaturated fatty acids such as linoleic acid (72–76%, w/w), which exceeds those in safflower oil (70–72%), sunflower oil (60–62%), and corn oil (about 52%)[7] (Ghisalberti, (15 March, 2001). Additionally, this oil is reported to contain minor components such as phenolic compounds.[8,9]

These compounds are reported to exhibit many biological activities, such as properties against the oxidation of low-density lipoproteins,[10] prevention of thrombosis[11] and health benefits for cardiovascular and non-alcoholic fatty liver disease.[12,13]

The traditional way to obtain oil from seeds is by their extraction with organic solvents, which is liable to introduce toxic solvent residues.[14] The application of Supercritical Fluid Extraction (SFE) is a promising technology for extraction of edible oils containing labile fatty acids avoiding the use of solvents.[15] This technique uses carbon dioxide as the solvent, and allows the design of environment-friendly processes, the processing of biological materials (carbon dioxide has a near-ambient critical temperature), and the possibility of obtaining products free of solvent residuals.[16] In addition, the solubility and selectivity of fatty acid oils can be optimized by adjusting the supercritical fluid carbon dioxide pressure and temperature conditions.

Several researchers have studied the SFE of oils from different plant seeds.[17,18] However, only a few of these studies have been focused on the extraction of oil from grape seeds.[19,20] Therefore, the aim of this work was to propose a method to obtain an extract rich in health promoting compounds by using SFE. To that end two temperatures were explored (40 and 60ºC) combined with different pressures conditions (200, 250 and 300 bar). The oils with higher content of bioactive compounds were used to determine their antioxidant and anti-inflammatory activities.

Results and discussion

Supercritical Fluid Extraction

SFE temperature and pressure conditions were explored for bioactive compounds extraction. Considerable variation in fatty acid concentration was observed according to the conditions applied. Conditions were selected on the basis of the extraction yield of

the oil collected, fatty acid profile and total phenolic content. The extraction yield was calculated as the ratio between the mg of oil produced and the grams of grape seeds used. Table 1 shows the yield obtained with each one of the conditions applied. The total oil content ranged from 8.51 to 220.34 mg/g of grape seeds. The highest yield was recovered when 40 ºC and 300 bar or 60 ºC and 250 bar were used. The fatty acid profile and total phenolic content found in the extract of grape seeds are illustrated in Table 2. It was observed that under 40 ºC and 300 bar it was also possible to extract the highest variety of fatty acids and higher concentration of total phenolic compounds. Based on these considerations 40 ºC and 300 bar were selected to carry out the extractions.

Fatty acid composition

As can be seen in Table 2 the fatty acid content ranged from 0.05 to 72.45%. It was possible to observe the presence of myristic acid (C14:0) when 300 bar and 40 ºC were used in the SFE. However myristic acid wasn´t detected at any other of the conditions considered. Linolenic acid (C18:3) was obtained in the conditions at 40 ºC whatever the pressure was set. Nevertheless, when the temperature applied was 60 ºC it was only extracted at 300 bar.

The lipid fraction of grape seeds was mostly composed by linoleic acid (C18:2, from 68.31 to 72.45%), followed by oleic acid (C18:1, from 15.35 to 17.98%), palmitic acid (C16:0, from 7.29 to 7.69%) and stearic acid (C18:0, from 5.06 to 4.01%). In addition to these main fatty acids others were also detected, namely: myristic (C14:0), palmitoleic (C16:1), heptadecanoic (C17:0), α-linolenic (C18:3), arachidic (C20:0), and eicosenoic (C20:1). Similar fatty acid profile in grape seed oil has been reported by other authors.[21]

Some of these fatty acids have demonstrated health benefits for different diseases. Particularly, linoleic acid has demonstrated anticarcinogenic effects, and as precursor of eicosanoids, has shown anti-inflammatory and antithrombotic activities.[22,23] Oleic acid has been reported to influence the induction of autophagy and to exert effect on apoptosis,[24] whereas, in the same study, palmitic acid was able to suppress the autophagy and is important in inducing apoptosis.

When considering the general classification of the fatty acids, it was found that grape seeds oils had the following sequence: PUFAs>MUFAs>SFAs, which is in agreement with other studies.[25] Taking into account the high proportion of unsaturated fatty acids in grape seed oil (~ 70%) and their health benefits[25] they can be recommended for human consumption, presenting a more favourable fatty acid profile than other vegetable oils.

Phenolic composition

The Total Phenolic Composition (TPC), obtained for the samples extracted at different SFE conditions, is presented in Table 2. It was observed a trend to increase the TPC with pressure. The lowest TPC value was observed at 200 bar and 60 ºC (3 ± 1 mg GAE/g), while the highest TPC was obtained at 300 bar and 40 ºC (25 ± 3 mg GAE/g). The TPC data obtained by SFE at 300 bar were similar to that observed by Kornsteiner *et al.,*[26] for almonds with skin (23 mg GAE/g).

The extract obtained applying the SFE selected conditions was subjected to HPLC analysis. Five phenolic acids were identified and quantified (Table 3). Gallic acid was determined at higher concentrations followed by caffeic acid and ρ-coumaric, whereas ferulic acid and ellagic acid were the phenolic acids present at lower concentration. Other authors have reported these compounds in grape seeds.[27,28] Differences in the

156 composition of the extracts were mainly due to the extraction conditions and the
157 techniques used in the extraction process.

158 **Antioxidant activity**

159 The antioxidant activity of grape seed oil was determined through the $DPPH^{\cdot}$ and
160 $ABTS^{\cdot+}$ radicals scavenging assays (Figure 1). Both assays have been applied to the
161 evaluation of the total antioxidant activity of various substances and widely used in
162 many recent studies for the detection of the total antioxidant activity of edible vegetable
163 oils.[32] As can be seen in the Figure, DPPH and ABTS activities increased with the
164 concentration. They varied from 29.2% at 12.5 μg/mL to 95.8% at 200 μg/mL when the
165 extracts were evaluated using the DPPH assay. The oils exerted significantly higher
166 antioxidant activities in the ABTS assay (91.4% at 100 μg/mL).

167 It is well established that $DPPH^{\cdot}$ radical is used to evaluate the free radical scavenging
168 activity of hydrogen donating antioxidants. $ABTS^{\cdot+}$ in addition measures the chain
169 breaking antioxidants.[29] Based on the above considerations, our results suggest that the
170 grape seed oil extracts are potent free radical scavenger and may be utilized as a good
171 source of natural antioxidants to be used as both nutraceutical and functional food
172 ingredients. Our findings seemed to be in good agreement with previous studies
173 reporting the antioxidant activity of extracts from different varieties of grape seeds.[25]

174 **Nitrite inhibition**

175 Several studies have shown that grapes may have anti-inflammatory properties. In this
176 line, grape extracts have demonstrated higher anti-inflammatory activity when
177 compared to the commercial non-steroidal anti-inflammatory drug (NSAID)
178 indomethacin.[30] *In vivo* studies have been also carried out demonstrating the
179 suppression effect of extracts obtained by red and white grape pomaces on chronic
180 inflammation induced by lipopolysaccharide and galactosamine.[31] The authors found

that the extract of red grape pomace suppresses the activation of inflammatory transcription factor NF-κB. All these studies consider grape samples rich in polyphenols. However, although the anti-inflammatory properties of some berry fatty acids have been reported by some authors[32] the inflammatory inhibition effects of grape seed fatty acids have not been considered to date.

In this study we evaluated the ability of grape seeds extracted by SFE to inhibit LPS-induced nitric oxide production in Raw 264.7 macrophages. First, the effect of the extracts on cell viability was examined at various concentrations. Exposure of the cells to the extracts (12.5, 25 and 50 µg/ml, for 24 h) did not show any significant cytotoxic effect in the present experiments (data not shown).

When cells were incubated with LPS the NO production increased by eight fold (Figure 2). The effects of the seed extracts did not show any decrease on the NO release at 12.5 and 25 µg/ml. However when 50 µg/ml were used a significant decrease was observed. Considering that NO is a late inflammatory marker formed through activation of inducible nitric oxide synthase (iNOS) its inhibition plays an import role for anti-inflammatory candidates.

The results presented here show that the SFE grape seed extracts are able to decrease inflammation *in vitro* by inhibiting LPS-induced NO in macrophages. These results are in agreement with the antioxidant activity shown above, thus indicating that the grape seeds bioactive compounds are responsible for these activities.

This is consistent with previous studies that reported that the antioxidant potentials of plant oils can be attributed to the PUFAs and the phenolic compounds.[6,21] In this line, Jiao *et al.*[33] studied two methods of extraction of pumpkin seed oil and evaluated their antioxidant capacity. They reported higher antioxidant activity of the oil with higher amount of PUFAs, according to the DPPH radical scavenging assay and the ß-

carotene/linoleic acid bleaching test. They also found that among the fatty acids identified, the oil produced by this method had higher amount of linoleic acid.

Other authors have associated the antioxidant and anti-inflammatory capacity of plant seeds with their phenolic content. In a manuscript published by Fazio *et al.*[34] they evaluated the anti-inflammatory and antioxidant activity of the seeds of *Sambucus* and *Rubus* species. The results showed that *Rubus* seeds had higher activities. These effects were correlated to the total phenolic content of the seeds.

As a summary of overall results, SFE extraction has shown to be an effective method to obtain health promoting bioactive compounds from grape seeds. The results of our study showed that grape seed extracts possess significant free radical scavenging activity and the ability to inhibit nitrite production. According to that, grape seeds are a valuable source of natural antioxidant and anti-inflammatory extracts for optimal human health.

Considering that seeds are obtained as a residue during wine and grape juice production, the SFE method here developed is proposed as a mean to obtained an added value product to be used as a nutraceutical or functional food ingredient by pharmacological and food industries. These results encourage further studies in order to scale up the process for possible industrial production of high quality bioactive ingredients.

Experimental

Chemicals and reagents

Ethanol and methanol were obtained from Carlo Erba Reagenti (Milan, Italy). The methyl esters of miristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) and arachidic acids were purchased from Sigma-Aldrich (Milan, Italy). Sodium metoxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,6-

231 di-*tert*-butyl-4-methylphenol (BHT), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-
232 carboxylic acid (Trolox), were also acquired from Sigma-Aldrich (Milan, Italy). The
233 supercritical fluid used was carbon dioxide (CO_2 > 99% purity) from Contse (Madrid,
234 Spain). 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt was from
235 TCI-Ace (Tokyo, Japan). Griess reagents and nitrite standard were supplied by Cayman
236 Chemical (Ann Arbor, MI, USA). Azobis(2-amidinopropane) dihydrochloride (AAPH)
237 was obtained from Wako Chemicals USA Inc. (Richmond, VA). Table red globe grapes
238 from two different batches were obtained from a local market. Oil was extracted from
239 the seeds by SFE.

240 **Supercritical Fluid Extraction**

241 Extractions were carried out using an SFE pilot plant designed and manufactured by
242 PID Eng. & Tech (Colmenar Viejo, Madrid, Spain). It consists of an extraction module
243 and two separation modules, which can allow fractionating the extracted products. Each
244 module includes process system and electronic control unit. The extraction system was
245 fully automated and controlled by using the Adkir software (PID Eng & Tech). It is
246 equipped with a Dosapro Milton Roy carbon dioxide pump, which can operate up to 75
247 mL/min and 380 bar and a Dosapro Milton Roy co-solvent pump. The system is fitted
248 with a 350 cc classic filter vessel extraction cell for solid sample, porous plate 20
249 microns, and quick connectors for easy work. A coiled heater allows the temperature
250 control of the extraction (internal thermocouple). Seeds were ground using a centrifugal
251 mill (Moulinex, Allençon, France) and passed through 60-mesh sieve with 0.5 mm pore
252 size. In each experiment, 6 g of milled and sieved seeds were placed inside the
253 extractor. The solvent used was carbon dioxide and the solvent flow rate applied was
254 1.5 l/min. Two levels of temperature were used (40 and 60 ºC) combined with different
255 pressures (200, 250 and 300 bar). The extractions were done in triplicate. The extracted

oils were stored, under enriched carbon dioxide atmosphere and protected from light, at -20 ºC until further analyses. Conditions were selected based on the oil mass yield, fatty acid composition and total phenolic content of the oil.

Fatty acid methyl esters derivatization method

Transesterification of extracted fatty acids from grapes to fatty acid methyl esters (FAMEs) was carried out based on the method previously proposed by Ruiz del Castillo *et al.*[35] Briefly, 100 mg of sample was mixed with 2.0 mL of MeOH and 2.0 mL of 0.5 N sodium methoxide. The mixture was heated at 50 °C on a heating block for 10 min. Subsequently, 100 µL of glacial acetic acid, 5 mL of saturated sodium chloride, and 3 mL of MeOH containing butylated hydroxytoluene (BHT; 50 ppm) were added. After shaking the tube and centrifuging the contents, the upper layer was removed and put through an anhydrous sodium sulfate column. The sample was then ready for analysis by gas chromatography (GC).

Fatty acid composition

Fatty acids were analyzed in a gas chromatograph (GC) (Hewlett-Packard model 6890), equipped with a split/splitless injector system and a flame ionization detector (FID). The GC separations were performed on a 25 m x 0.25 mm i.d. capillary column coated with a 0.25 µm layer of polyethylene glycol (007 Carbowax 20M, Quadrex). Helium was used as carrier gas at a constant pressure on head column of 25 psi. Injector and detector temperatures were 250 °C and 300 °C, respectively. The injector was operated in the splitless mode. The GC column was initially programmed at 4 ºC/min from 70 ºC to 230 ºC (5 min) and detection was performed with a flame ionization detector (FID). Data acquisition was accomplished with the HP-ChemStation system. The identification of the chromatographic peaks was made by comparison with the retention time of the sample peaks and those of fatty acid methyl ester standards injected.

Total phenol content

Total phenol content was assessed by Folin-Ciocaltue method.[36,37] Three aliquots were analysed in triplicate (n = 9). To 100 µL of sample or gallic acid, 1mL of Folin-Ciocaltue reagent was added, mixed and incubated for 5 min at room temperature prior to addition of 1mL of 0.1 mmol/L Na_2CO_3 solution. This mixture was then allowed to stand for 90 minutes at room temperature, and the absorbance was determined at 765 nm. Total phenolic content was estimated as gallic acid equivalents (GAE, mg gallic acid/g dry fruit material).

HPLC-DAD analysis

A Konik (Konik, Sant Cugat del Valle´ s, Barcelona, Spain) liquid chromatography system model 560 equipped with a Konik 560 UV–Vis detector, a 20 µL sample loop and a column thermostat was used. Separation was carried out on a 250 x 4.6 mm, 5 µm ODS reverse phase (C18) column (ACE, Madrid, Spain). The elution solvents A (1% aqueous formic acid solution) and B (60% MeOH with 0.5% formic acid) were applied as follows: flow rate, 1 mL/min; isocratic 95% B for 10 min, from 95–90% over 15 min, from 90–85% over 10 min and from 85–75% over 20 min. The composition was then changed to initial condition in 5min, and maintained for 10 min. Stock solution of gallic acid, caffeic acid, ρ-coumaric acid, ferulic acid, and ellagic acid were prepared in 70% (v/v) methanol to final concentration of 1mg/mL. Each stock solution was further diluted to obtain six concentrations of the standard for HPLC–PDA quantification and they were injected in triplicate. The oil extracts were filtered and injected in triplicate.

Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH$^{\bullet}$) radical scavenging assay

The DPPH assay was carried out according to the method developed by Smith *et al.*[38] with minor modifications. Briefly, 150 µL of DPPH in ethanol (400 µM) and 50 µL

aliquot of the sample in different concentrations (12.5, 25, 50, 100 and 200 µg/mL) were added 96-well microtiter plate. Decrease of absorbance was monitored at 517 nm after 30 min of incubation at 37 ºC on a microplate reader (Biotek Instruments, Winooski, VT, USA). The percentage inhibition of the DPPH by each dilution of samples was calculated considering the percentage of the steady DPPH in solution after reaction.

ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity

The ABTS radical scavenging capacities of the grape-seed oils was determined based on the method of Re *et al.*[39] Briefly, the $ABTS^{\cdot+}$ radical was prepared by the reaction of the ABTS aqueous solution (7 mM) with $K_2S_2O_8$ (2.45 mM, final concentration) at ambient temperature in the dark for 12–16 h. The $ABTS^{\cdot+}$ solution was then diluted with ethanol to obtain an absorbance of 0.700 (±0.20) at 734 nm. In a final volume of 200 µL, the reaction mixture compromised 190 µl of the $ABTS^{\cdot+}$ solution and 10 µL of the grape seed oil at different concentrations (.5, 25, 50, 100 and 200 µg/mL). Absorbance was measured 6 min after mixing. The percentage inhibition for different concentrations was calculated by determining the percentage of the steady ABTS in solution after reaction.

Cell Viability Assays

Cell viability assay was performed using the MTT photometric analysis and trypan blue dye exclusion method. Briefly, cells were plated in wells of 96-well plates and incubated for 24 h at 37 ºC. Cells were treated with or without the extracts. At the end of the treatment, the medium was removed and cells were incubated with 100 µl of MTT (5 mg/ml in phosphate buffered saline; PBS) in fresh medium for 4 h at 37 ºC. After 4 h, formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in DMSO (150 µl/well). After mixing, the absorbance of the cells was

measured at 540 nm following 10 min of incubation on the iMark Microplate Reader BioRad (Hercules, CA, USA). The dye exclusion method was performed by calculating the number of died cells over the control.

Nitrite Quantification

The content of nitrite, one of the end products of NO oxidation, was monitored by a procedure based on the diazotidation of nitrite by sulfanilic acid (Griess reaction). Twenty-four hours after the incubation of Raw 264.7 cells with 0.4 µg/ml of LPS, 50 µL of sample aliquots were mixed with 50 µL of Griess reagent in 96-well plates and incubated at room temperature for 15 min. The absorbance (520 nm) of the mixture was measured on a microplate reader. The concentration of nitrite was calculated with the linear equation derived from the standard curve generated by known concentrations of sodium nitrite.

Statistical Analysis

Data are expressed as means values ± 95% confidence interval. Analysis of variance was performed by one-way analysis of variance (ANOVA) with significant differences between means determined by the Student's t-test. JMP Statistics software package version 8 was used for statistical analyses (SAS Institute Inc., NC, USA).

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Figure Captions

Figure 1. DPPH a) and ABTS b) radical scavenging activity (%) of grape seed extracts at different concentrations (12.5, 25, 50, 100 and 200 µg/mL)

Figure 2. Production of extracellular nitrite in Raw 264.7 cells stimulated with LPS (0,4 lg/mL) for 24 h in the presence of grape seed extracts at 12.5, 25 and 50 µg/mL. Data are expressed as the mean ± SD from two independent experiments and quantified using Griess reagent. Bars different letters (a–c) are significantly ($P > 0.05$).

Table 1. Extract oil yield at different SFE temperature and pressure conditions

Temperature (ºC)	Pressure (bar)	yield (mg/g grape seeds)
40	200	8,5
40	250	82,1
40	300	220,3
60	200	155,3
60	250	216,1
60	300	150,8

Table 2. Fatty acid composition and total phenolic content of grape seeds extracts.

Fatty acids	Concentration of fatty acids in % of ∑ FAME					
	200 bar 40ºC	250 bar 40ºC	300 bar 40ºC	200 bar 60ºC	250 bar 60ºC	300 bar 60ºC
C14:0	–	–	0.08 ± 0.02	–	–	–
C16:0	7.58 ± 0.02	7.63 ± 0.01	7.42 ± 0.03	7.69 ± 0.01	7.34 ± 0.02	7.29 ± 0.01
C16:1	0.21 ± 0.04	0.20 ± 0.01	0.24 ± 0.01	0.29 ± 0.02	0.26 ± 0.01	0.25 ± 0.01
C17:0	0.06 ± 0.01	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
C18:0	4.97 ± 0.03	5.03 ± 0.01	4.03 ± 0.01	4.01 ± 0.03	4.57 ± 0.02	4.49 ± 0.02
C18:1ω9	17.85 ± 0.03	17.98 ± 0.03	17.49 ± 0.02	15.35 ± 0.01	16.41 ± 0.01	15.93 ± 0.01
C18:2ω6	68.31 ± 0.01	68.01 ± 0.01	69.78 ± 0.03	72.45 ± 0.03	71.14 ± 0.02	71.68 ± 0.02
C18:3ω3	0.78 ± 0.01	0.79 ± 0.02	0.56 ± 0.03	–	–	0.06 ± 0.02
C20:0	0.11 ± 0.02	0.17 ± 0.03	0.20 ± 0.01	–	0.21 ± 0.02	0.09 ± 0.01
C20:1ω9	0.13 ± 0.01	0.12 ± 0.02	0.15 ± 0.03	0.15 ± 0.02	–	0.14 ± 0.01
Σ SFA	12.72 ± 0.03a	12,90 ± 0.03b	11.78 ± 0.03c	11.76 ± 0.03c	12.19 ± 0.02d	11.94 ± 0.03e
Σ MUFA	18.19 ± 0.02a	18.30 ± 0.02b	17.88 ± 0.02c	15.79 ± 0.03d	16.67 ± 0.03e	16.32 ± 0.02f
Σ PUFA	69.09 ± 0.03a	68,80 ± 0.03b	70.34 ± 0.03c	72.45 ± 0.02d	71.14 ± 0.03e	71.74 ± 0.03f
TPC (mg GAE/g)	7 ± 3a	17 ± 4b	25 ± 3c	3 ± 1a	10 ± 2a	16 ± 3b

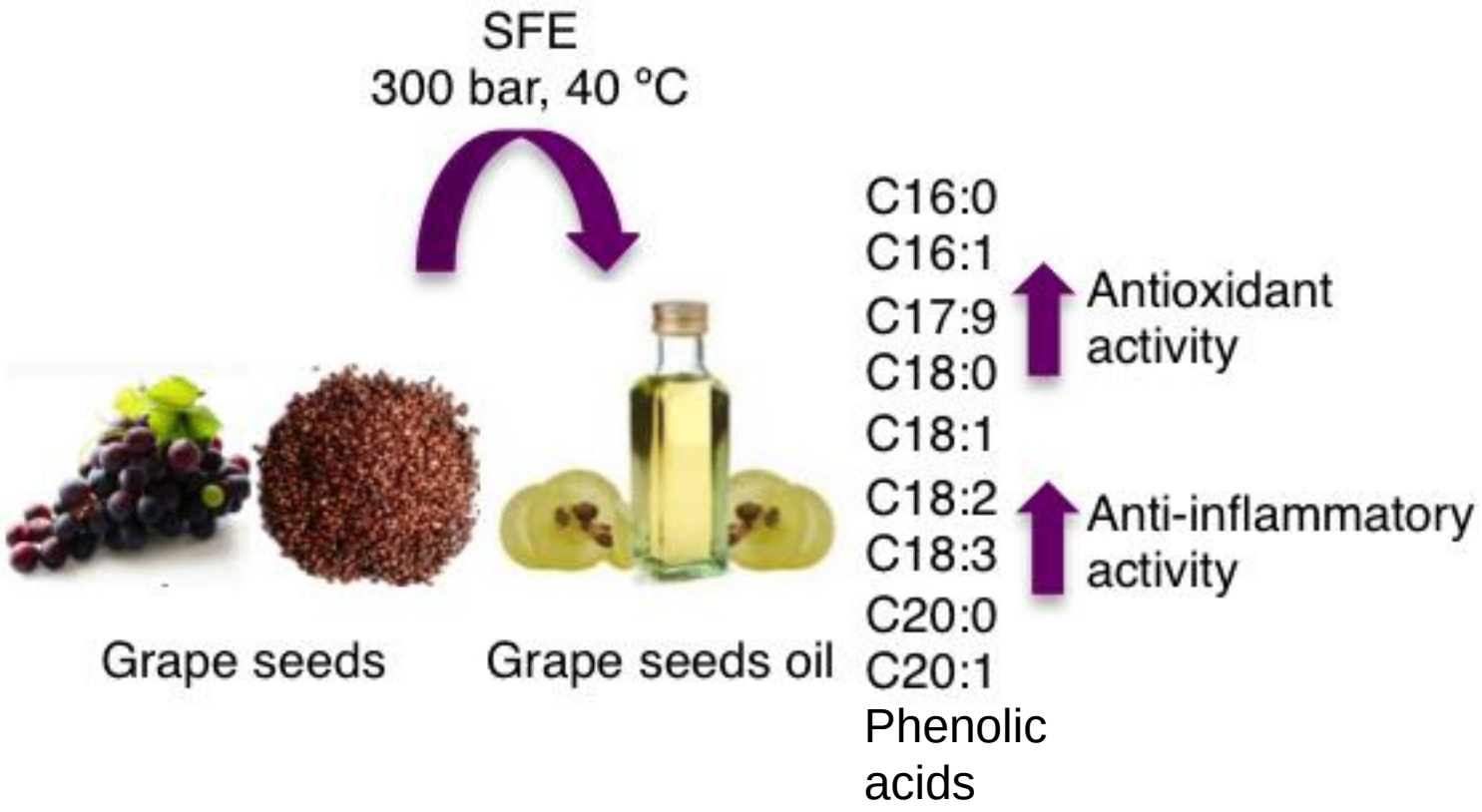
C14:0 (myristic acid); C16:0 (palmitic acid); C16:1 (palmitoleic acid); C17:0 (heptadecanoic acid); C18:0 (stearic acid); C18:1 (oleic acid); C18:2 (linoleic acid); C18:3 (linolenic acid); C20:0 (arachidic acid); C20:1 (eicosenoic acid); SFAs (saturated fatty acids); MUFAs (monounsaturated fatty acids); PUFAs (polyunsaturated fatty acids).

Same letters indicate no significant difference at level of 5% ($p < 0.05$)

Table 3. Concentration of identified phenolic acids (g/kg of extract) in the grape seed oil extracted at 40ºC and 300 bar

Compound	Concentration
Gallic acid	2.01 ± 0.19a
Caffeic acid	0.42 ± 0.09b
ρ-Coumaric acid	0.22 ± 0.03c
Ferulic acid	0.05 ± 0.01d
Ellagic acid	0.02 ± 0.01d

Same letters indicate no significant difference at level of 5% ($p < 0.05$)



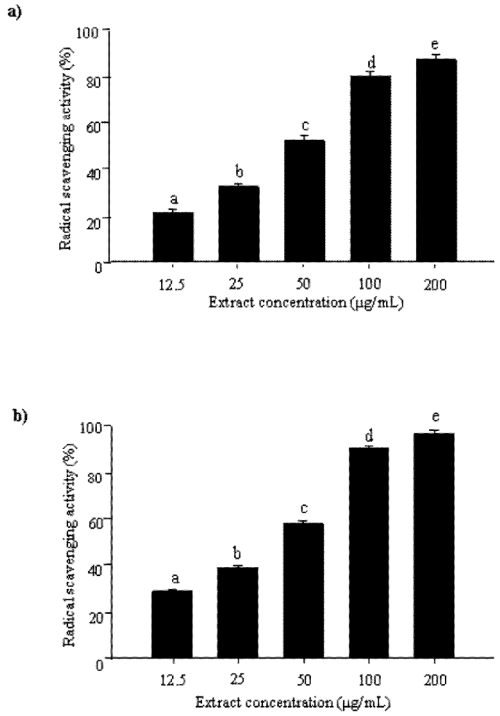


Figure 1
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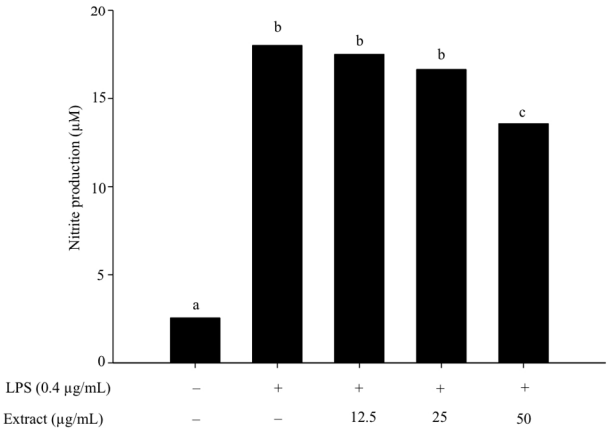

Figure 2
595x446mm (72 x 72 DPI)