INTRODUCTION

Grapes (Vitis sp., Vitaceae) are the second most important fruit crop worldwide, with more than 69 million tons produced in 2011 (FAO). Of these, about 80% are vinified each year, generating approximately five to nine million tons of residues. These by-products are difficult to dispose of and sometimes may represent a serious environmental problem (Schieber et al., 2001). An alternative is their processing as an abundant and cheap source of phenolic compounds, widely appreciated for their health and nutritional properties (Iacopini et al., 2008). In this sense, one of the possibilities is the use of fresh and dried grape skins as an infusion due to their nutraceutical and health-protecting properties (Cheng et al., 2010). However, to avoid potential health problems, the biological activity of these grape skin by-products should be carefully characterised using in vitro and in vivo models (Iriti & Faoro, 2009).

Polyphenols obtained from grapes and grape skins are normally separated into two principal groups: non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and stilbenes) and flavonoids (flavan-3-ols, flavonols and anthocyanins) (Shi et al., 2003). It has been demonstrated widely that their phenolic composition and range depend, among other factors, on the grape variety (Kammerer et al., 2004), the grape growing and maturation conditions (Ojeda et al., 2002), the winemaking methodology employed (Sacchi et al., 2005) and the treatment to obtain the skins (Rodríguez Montealegre et al., 2006). These phenolic compounds showed an impairing capacity against the three steps of carcinogenesis by acting at multiple levels (Signorelli & Ghidoni 2005). Their activity was observed in inhibiting phase I enzymes (avoiding damage at DNA level, the first step) (Chang et al., 2001), downregulating the expression of DNA methyltransferases and PGE2 cancer-promoting prostaglandin (Zhu et al., 2012a) and modulating the mitogenic signalling and cell growth (the second step), and inducing apoptosis and cellular G1 arrest (the third step) (Agarwal et al., 2000).

One of these major phenolic compounds observed in most of the wine by-products is the resveratrol (3,4’,5-trihydroxy-trans-stilbene), a phytoalexin that showed DNA-protective effects against H2O2-induced damage and cytotoxic effects in in vitro and in vivo assays (Rotondo et al., 1998; Baur & Sinclair, 2006). In addition, resveratrol is recognised by its ability to prevent protein oxidation and platelet aggregation and to inhibit cyclooxygenases (Subbaramiah et al., 1999). Furthermore, this compound is also widely studied as a promising nutraceutical molecule with a key role against carcinogenesis and cardiovascular diseases (Signorelli & Ghidoni, 2005; Ruan et al., 2012).

However, all new complex mixtures with a potential use as nutraceuticals must be tested against possible mutagenic and toxic effects. For this purpose, one of the most employed methodologies is the somatic mutation and recombination
test (SMART), performed in imaginal discs of the *Drosophila melanogaster* larvae. The so-called “wing spot test” is a reliable procedure to evaluate the genotoxicity of single compounds and complex mixtures due to the bio-activation competence observed in the larvae (Graf et al., 1994). With this *in vitro* methodology, we analysed the capability of grape extracts to inhibit the mutagenicity induced by a genotoxic oxidative model (H$_2$O$_2$).

Furthermore, the cytotoxic and pro-apoptotic effects of these phenolic compounds against the carcinogenic process were determined employing the human promyelocytic leukaemic (HL60) cell *in vitro* model (Birnie, 1988). This methodology was widely employed by our group to quantify the beneficial effects of several natural mixtures belonging to the “Mediterranean diet” against cancer development in humans (Anter et al., 2011; Anter et al., 2014).

Therefore, the aims of this study were: 1) to characterise the phenolic compounds present in two different grape skin extracts obtained from Spanish wineries and 2) to assess the biological activity of grape skin extracts and resveratrol regarding their role in the maintenance of genetic stability and their potential chemopreventive effects as a required step before their potential use in the nutraceutical industry.

**MATERIAL AND METHODS**

**Samples**

Fresh grape skins and skins from the vinification marcs were collected from Syrah red grapes (*Vitis vinifera*) grown in the Andalusian region of Sierra de Segura, Spain. Fresh grapes were de-stalked and the skins were removed manually. Grape skins from vinification residues (marcs) were obtained directly in the winery after the grape juice had been collected after the initial winemaking maceration.

**Microwave-assisted extraction (MWE)**

The working conditions for the extraction of the target compounds from grape skins were: 12.5 g of the raw material extracted with 100 mL 60% (v/v) aqueous ethanol at pH 4. Microwave irradiation at 140 W was applied for 10 min according to our laboratory method (Pérez-Serradilla & Luque de Castro, 2011).

**Analytical methods**

All the chemical determinations and characterisations that are reported in this manuscript were carried out in the laboratory for metabolomics/proteomics and the exploitation of agricultural food residues at the University of Córdoba. Unless a different brand is described, reagents were purchased from Sigma Aldrich, Spain. More information is available in the supplementary information at the end of this article.

**Determination of total phenolic compounds by the Folin–Cioclateu (F–C) method**

Total phenol compounds in the extracts were quantified by the F–C method using gallic acid as standard. The results are expressed as equivalent to milligrams of gallic acid per mL of raw material extract (mg GAE/mL).

**HPLC analysis**

The extracts from the raw materials were dried in a rotary evaporator to half their initial volume to remove ethanol. To avoid any loss of volatile phenols, the entire evaporation process was carried out at a controlled temperature of 20 °C. After that, all extracts were centrifuged for 10 min at 850 g to separate the solid residue from the extracts. Finally, each extract was filtered using a 0.45 µm filter before injection into the chromatograph.

Individual separation of phenols in the extract was performed on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 µm particle, Análisis Vinicos, Tomelloso, Ciudad Real, Spain), using an injection volume of 20 µL and a flow rate of 1 mL/min. Mobile phase A consisted of 0.2% (v/v) phosphoric acid aqueous solution and mobile phase B consisted of methanol. The gradient method was as follows: from 96% to 82% A in 20 min, held for 20 min, from 82% to 74% A in 24 min and from 74% to 50% B in 9 min. The analytes were identified by comparing both their retention times and UV spectra with those of the corresponding standards. The absorption wavelengths were set at 260 nm for monitoring ellagic acid; at 280 nm for hydroxybenzoic acids, catechin and phenolic aldehydes; at 320 nm for hydroxycinnamic acids, and at 360 nm for hydroxycinnamic aldehydes.

**Genotoxicity and antigenotoxicity assays (SMART)**

**Strains**

Two *Drosophila* strains were used: (i) *mwh/mwh*, carrying the recessive mutation *mwh* (*multiple wing hairs*) that produces multiple tricomas per cell (Yan et al., 2008); and (ii) *flr*/ *In (3LR) T3M, ri p′ sep bx^{te} c′ B^d*, where the *flr* (*flare*) marker is a homozygous recessive lethal mutation viable in homozygous somatic cells that produces deformed tricomas (Ren et al., 2007). Detailed information on the rest of the genetic markers is available in Lindsley and Zimm (1992).

**Treatments**

The SMART assay, developed by Graf et al. (1984), was carried out following our standard procedure (Anter et al., 2014). Briefly, *mwh/mwh* males and *flr*/ *T3M* virgin females were allowed to mate for two days. After eight hours of egg laying, the 72h-transheterozygous synchronised larvae were reared until pupation in glass vials containing 0.85 g of *Drosophila* Instant Medium (Formula 4-24, Carolina Biological Supply, Burlington, NC), supplemented with the tested compounds at two different concentrations: Fresh grape skin extracts (FGSE: 1.25 to 20 µL/mL), vinification grape skin extracts (VGSE: 1.25 to 20 µL/mL) and resveratrol (33 to 528 µM). The range of resveratrol concentrations employed corresponds to the content determined in the extracts (Table 1). Concurrent negative (H$_2$O$_2$) and positive (H$_2$O$_2$ + resveratrol) bioassays were carried out for each treatment to determine the percentage of larvae with at least one multiple wing hair (mwh) or heterozygous somatic cells that produces deformed tricomas.

**Determination of total anthocyanin content**

The total concentration of anthocyanins in the extracts was estimated spectrophotometrically by monitoring the absorbance of the extract at 535 nm, the selective wavelength for the target compounds in the extracts. The concentration of anthocyanins was expressed in mg equivalents of P3G/g (mg P3G/g) of dry weight.

**Determination of anthocyanins on HPLC**

The anthocyanins in the extracts were quantified using a Merck 6460D diode array detector (DAD) with the same HPLC method described above (280 nm) for monitoring ethyl gallate; at 320 nm for hydroxycinnamic acids, and at 360 nm for hydroxycinnamic aldehydes.
TABLE 1
Phytochemical profile of fresh grape skin extract (FGSE) and vinification grape skin extract (VGSE).

<table>
<thead>
<tr>
<th>Target compound</th>
<th>Concentration in fresh grape skin extracts (FGSE)$^{(1)}$</th>
<th>Concentration in vinification grape skin extracts (VGSE)$^{(1)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid</td>
<td>0.57 ± 0.00</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>Coumaric acid</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>4.39 ± 0.00</td>
<td>5.40 ± 0.07</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>101.0 ± 0.2</td>
<td>21.99 ± 0.6</td>
</tr>
<tr>
<td>Myricetin</td>
<td>5.57 ± 0.05</td>
<td>2.15 ± 0.06</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.89 ± 0.02</td>
<td>1.18 ± 0.01</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.30 ± 0.00</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>Total anthocyanin content$^{(2)}$</td>
<td>97.57 ± 0.04</td>
<td>43.47 ± 0.03</td>
</tr>
<tr>
<td>Total phenolic content$^{(3)}$</td>
<td>1555.33 ± 0.05</td>
<td>1616.82 ± 1</td>
</tr>
</tbody>
</table>

Notes: $(1)$ Expressed as mg/mL. $(2)$ Expressed as mg equivalents of peonidin-3-glucoside/g (mg P3G/g) of dry weight. $(3)$ Expressed as equivalent to milligrams of gallic acid per mL of raw material extract (mg GAE/mL). n.d. = no data

controls (0.12 M hydrogen peroxide, Sigma, H1009) were run.

The antigenotoxicity test was performed following the method described by Graf et al. (1998). Larvae were reared following the same protocol described previously in co-treatment with 0.12M H$_2$O$_2$ as mutagenic agent.

Upon hatching, flies were fixed in 70% ethanol and the mwh/flr$^{3}$ wings were mounted on slides and analysed using a 400 x bright field microscope. Both the ventral and dorsal surfaces of the wings were analysed. Mutations were characterised as small single spots (one or two cells), large single spots (more than two cells) of either mwh or flr$^{3}$, and twin spots (mwh-flr$^{3}$). Small and large spots can originate from somatic point mutation, chromosome aberration as well as somatic recombination, whilst twin spots are produced exclusively by somatic recombination between the flr$^{3}$ locus and the centromere.

**Data evaluation and statistical analysis**

The treatment series were compared with concurrent water control using the multi-decision procedure described by Frei and Wurgler (1995) to determine whether the result was positive, inconclusive or negative.

In the co-treatments with H$_2$O$_2$, the inhibition percentage of FGSE, VGSE and resveratrol for total spots was calculated as described by Abraham (1994): $P = \frac{(TSG - TSGP)}{TSG} \times 100$

where $TSG = \text{total spots of genotoxin alone}$; $TSGP = \text{total spots of genotoxin and tested product}$

**Cytotoxicity assays**

**Cell culture**

Cells were cultured in RPMI 1640 medium (Biowhittaker, BE12-167F), supplemented with 10% heat-inactivated bovine serum (Biowhittaker, DE14-801F), 200 mM L-glutamine (Sigma, G7513) and an antibiotic-antimitotic solution with 10 000 units of penicillin, 10 mg of streptomycin and 25 µg amphotericin B per mL (Sigma, A5955), using tissue culture plastics from Techno Plastic Products AG (Switzerland). Cells were grown at 37°C in a humidified 95% air–5% CO$_2$ atmosphere and sub-cultured three times per week.

**Assessment of cell viability**

HL60 cells were placed in 12-well culture plates at 2 x 10$^5$ cells/mL and treated for 72 h with different concentrations of each treatment with 0.12 M hydrogen peroxide, Sigma, H1009, and resveratrol (5 to 20 µL/mL and 33 to 528 µM respectively). The cell viability was assessed by the trypan blue exclusion method following our standard procedure (Anter et al., 2011). The percentage of unstained (viable) and stained (dead) cells and the IC$_{50}$ values were determined in three independent replicates.

**Analysis of DNA fragmentation**

Suspension cultures of HL60 cells (1.5 x 10$^6$ cells/well) were treated with FGSE (1 to 20 µL/mL), VGSE (1 to 20 µL/mL) and resveratrol (33 to 528 µM) for 5 h. The cells were centrifuged at 956 g for 5 min and washed with phosphate-buffered saline solution. The DNA was extracted using a commercial DNA extraction kit (Dominion mbl, MBL 243), and treated with RNase. A final amount of 1 500 ng of DNA of each treatment was resolved by electrophoresis at 50 V/cm for 120 min on 2% agarose gel impregnated with ethidium bromide. A DNA molecular weight reference (Dominion mbl, MBL 021) was run in parallel and DNA fragments were visualised under UV light.

**RESULTS AND DISCUSSION**

**Phenolic content of grape skin extracts**

Table 1 shows the phytochemical profile of fresh grape skin extract (FGSE) and vinification grape skin extract (VGSE). Our results showed a higher total phenolic content (1 555.33 mg in FGSE and 1 616.82 mg in VGSE) and total anthocyanin content (97.573 in FGSE and 43.473 in VGSE) in comparison with previous studies (Thimothe et al., 2007; Anastasiadi et al., 2012) performed in different cultivars. Furthermore, kaempferol (0.245 and 0.302 ppm) and total anthocyanin contents (43.47 and 97.57 mg P3G/g) were also higher than those reported previously by Zhu et al. (2012b).
and Muñoz et al. (2008). This could be due to the fact that phenolic content is strongly influenced by the grape variety, vintage year (Kallithraka et al., 2009) and extraction method (Delgado-Torre et al., 2012), and therefore the comparison between studies must be considered carefully. On the other hand, our results showed a much lower concentration of cyanidin (21.9 vs 101.0), myricetin (2.15 vs 5.57) and total anthocyanin content (43.473 vs 97.573) in VGSE compared to FGSE. This could be due to the fact that a large part of these anthocyanins (more than 50%) are transferred to the wine during maceration and winemaking (Van Balen, 1984), lowering the percentage observed in the by-products. In contrast, resveratrol (5.402 vs 4.388 ppm) and quercetin (1.185 vs 0.892 ppm) concentrations were higher in VGSE compared to FGSE. We suggest that these results could have been obtained due to the fact that the molecules migrate from the grape seeds and pomace to the skin by-products during winemaking, increasing their concentration. However, it is noteworthy that the differences observed were statistically not significant. Finally, resveratrol concentrations were much higher than those reported previously by Priego-Capote et al. (2007), who obtained only 0.71 µg/mL of grape skin extract. We speculate that the extraction procedure (Malovaná et al., 2001) and the different oenological practices and cultivars (Jeandet et al., 1995) could be responsible for these large differences, and consequently any attempt to trade these kinds of raw materials has to pass through a standardisation process.

### Genotoxic/antigenotoxic potential of FGSE, VGSE and resveratrol

Transheterozygous larvae of *Drosophila* were treated with FGSE (1.25 to 20 µL/mL), VGSE (1.25 to 20 µL/mL) and resveratrol (33 to 528 µM) to assess the lack of genotoxicity of these compounds on the genomic structure (Table 2). Hydrogen peroxide showed a mutation frequency of 0.57 with a significant increment of all spot categories when compared to the water control (0.27). Romero-Jiménez et al. (2005) showed that H$_2$O$_2$ is genotoxic in the SMART and increases single and multiple spots in *Drosophila melanogaster* due to genetic damage. In addition, H$_2$O$_2$ is an endogenous mutagen responsible for some of the most important cancer risks associated with persistent inflammations (Fitzpatrick, 2001). Oxy-radicals derived from H$_2$O$_2$ can act either directly on the genome, causing oncogenic mutations derived from chromosome damage (Burcham, 1999), or indirectly, by modulating gene transcription (Cerda & Weitzman, 1997) and suppressing genomic repair pathways (Hu et al., 1995; Ghosh & Mitchell, 1999). The genotoxic results for H$_2$O$_2$ validate it as an appropriate genotoxicant in SMART for screening between oxidative mutagens (positive controls as H$_2$O$_2$) and non-mutagens (distilled sterile water controls or potentially safe extracts).

Table 2 shows the results of genotoxicity assays for FGSE, VGSE and resveratrol. The three substances analysed were non-mutagenic, as the number of total spots per wing was not significantly different from the water control value (0.27). It is noteworthy that there are no previous studies evaluating vinification by-products using the SMART assay. However, these results agree with previous studies performed using different genotoxicity tests, like the *Salmonella/*microsome assay (Aiub et al., 2004) and those performed in laboratory rats (Lluis et al., 2011). These results are important in order to evaluate the use of this kind of by-products as a nutraceutical supplement. Furthermore, the tested compounds also counteracted the mutagenic effect of H$_2$O$_2$ in the antigenotoxicity test performed in *Drosophila melanogaster* larvae co-incubated with hydrogen peroxide (Table 3). Both grape skin extracts (FGSE, VGSE) and resveratrol showed negative results, indicating desmutagenic properties. These results are also in agreement with previous reports showing that these substances inhibit H$_2$O$_2$-induced mutagenicity, mainly by scavenging free radicals (Stagos et al., 2006). In this sense, a previous study also showed protective effects of commercial grape proanthocyanidins against the DNA damage induced by doxorubicin, a

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**TABLE 2**

*Summary of genotoxicity results obtained in the *Drosophila* wing spot test (SMART) for fresh grape skin extract (FGSE), vinification grape skin extract (VGSE) and resveratrol.*

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Number of wings</th>
<th>Small spots (1–2 cells) m = 2</th>
<th>Large spots (more than two cells) m = 5</th>
<th>Twin spots m = 5</th>
<th>Total spots m = 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$ (0.12M)</td>
<td>40</td>
<td>0.23 (9)</td>
<td>0.06 (2)</td>
<td>0</td>
<td>0.27 (11)</td>
</tr>
<tr>
<td>FGSE (mL/mL)</td>
<td>40</td>
<td>0.17 (7)</td>
<td>0.05 (2)</td>
<td>0</td>
<td>0.22 (9)</td>
</tr>
<tr>
<td>1.25</td>
<td>20</td>
<td>0.20 (8)</td>
<td>0.03 (1)</td>
<td>0</td>
<td>0.22 (9)</td>
</tr>
<tr>
<td>VGSE (mL/mL)</td>
<td>40</td>
<td>0.28 (11)</td>
<td>0</td>
<td>0</td>
<td>0.27 (11)</td>
</tr>
<tr>
<td>1.25</td>
<td>20</td>
<td>0.20 (8)</td>
<td>0</td>
<td>0</td>
<td>0.20 (8)</td>
</tr>
<tr>
<td>Resveratrol (mM)</td>
<td>33</td>
<td>0.15 (6)</td>
<td>0.03 (1)</td>
<td>0</td>
<td>0.17 (7)</td>
</tr>
<tr>
<td>528</td>
<td>40</td>
<td>0.15 (6)</td>
<td>0</td>
<td>0</td>
<td>0.15 (6)</td>
</tr>
</tbody>
</table>

*Statistical diagnoses according to Frei and Würgler (1988, 1995): + (positive), - (negative) and i (inconclusive). Significance levels * = P < 0.05, one-sided test without Bonferroni correction.*

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Effects of FGSE, VGSE and resveratrol on cell viability 

Cytotoxic and proapoptotic effects of fresh and vinification grape skin extracts were assayed in the HL60 human leukaemia cell line. Both compounds showed a high cytotoxic effect ($IC_{50} \approx 4.5 \mu M$) as well as resveratrol ($IC_{50} \approx 98 \mu M$) in a dose-dependent manner (Fig. 1). These results are in consonance with similar effects observed in HL60 using table grape skin extracts (Clément et al., 1998; Anter et al., 2011). It has been demonstrated that grape phenols can affect several metabolic pathways in cancer cells (King et al., 2006). Among them, apoptosis is more desirable for chemoprevention since it could be the mechanism with less side effects. As shown in Fig. 2, FGSE, VGSE and resveratrol, supplemented at high concentrations, induced a cleavage of chromosomal DNA into oligonucleosomal fragments, which is well known as an apoptosis marker. This induction of apoptotic cell death could explain the cytotoxic properties observed, supporting the hypothesis that the anticarcinogenic effects of FGSE, VGSE and resveratrol may be mediated by this particular pathway (Stervbo et al., 2006). In this sense, it has been reported that the apoptotic induction produced by resveratrol in HL60 cells is mediated mainly by increased caspase activity, leading to the occurrence of these characteristic morphological changes (Garvin et al., 2006). Furthermore, it has also been demonstrated that resveratrol can downregulate Bcl2 expression and upregulate Bax expression (Zhan et al., 1994; Roman et al., 2002), two major genes involved in the apoptotic pathway. Another possible explanation is the enhancement of p53 activity and expression via a Ras-MAPK kinase signal transduction pathway produced by resveratrol (Huang et al., 1999). Since the lack of p53 activity was pointed out as one of the main causes of chemotherapy resistance in cancer cells (Fisher, 1994), this improved activity could also be responsible for the pro-apoptotic effect against HL60 by FGSE and resveratrol observed in the present study.

CONCLUSIONS

Our results show some potential advantages of the use of FGSE and VGSE as food additives and/or nutraceuticals, as they exhibit pleiotropic biological effects and show three important biological capabilities: 1) they were characterised as non-mutagenic and desmutagenic in a robust in vivo model; 2) they show acceptable inhibition competences
Health-promoting Activity of Vinification By-products against oxidation-induced genetic damage and 3) they show an important cytotoxic and pro-apoptotic effect against a human cancer cell line. With this potential use of vinification waste, the pharmaceutical and/or nutraceutical industries could obtain an interesting and low-cost source of antioxidant compounds and wineries could reutilise and recycle the most important by-product generated during the commercial winemaking process.

SUPPLEMENTARY INFORMATION

(+)-Catechin, vanillin(4-hydroxy-3-methoxybenzaldehyde), quercetin(2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzoypuran-4-onedihydrate,3′,4′,5,7-pentahydroxyflavone dehydrate), kaempferol(3′,4′,5,7-tetrahydroxyflavone, 3,5,7-tri-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), caffeic acid(3,4-dihydroxyxycinnamic acid), resveratrol(3′,4′,5-trihydroxy-trans-stilbene, 5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol), cyanidin (3,3′,4,5,7-pentahydroxyflavylum chloride), myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone, cannabiscetin), syringaldehyde(4-hydroxy-3,5-dimethoxybenzaldehyde), coniferaldehyde(4-hydroxy-3-methoxyxycinnamaldehyde), sinapaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde), acetovanillone (1-(4-hydroxy-3-methoxyphenyl)ethanone), acetylsyringone(1-(4-hydroxy-3,5-dimethoxyphenyl)ethanone), 5-hydroxymethylfurfural(5-hydroxymethyl-2-furalcarboxaldehyde), pyrogallol(1,2,3-trihydroxybenzene), pyrocatechol(1,2-dihydroxybenzene), guaiacol (2-methoxyphenol) and gallic(3,4,5-trihydroxybenzoic acid), protocatechuic(3,4-dihydroxybenzoic acid), p-hydroxybenzoic, vanillic(4-hydroxy-3-methoxybenzoic acid), syringic(4-hydroxy-3,5-dimethoxybenzoic acid), p-coumaric(4-hydroxyxycinnamic acid), ferulic(4-hydroxy-3-methoxyxycinnamic acid), and synapic acids (4-hydroxy-3,5-dimethoxyxycinnamic acid) were from Sigma–Aldrich (St. Louis, USA), as was p-cresol (1-hydroxy-4-methylbenzene), used as external standard.

Apparatus

A Spectronic Helios Gamma Spectrometer (Termo Waltham, MA, USA) was used to monitor the absorbance of the extracts, and an F-2500 Hitachi Fluorescence Spectrophotometer (Pleasanton, Canada), equipped with a 10 mm path length cuvette, was used for monitoring the fluorescence in the ORAC assay.

Shaking and centrifugation of the extracts were carried out by means of an MS2 Minishaker (IKA, Germany) Vortex and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

A Microdigest 301 digester of 200 W maximum power, from Prolabo (Paris, France), was used to accelerate solid–liquid extraction.

Individual separation of the extract components was carried out by a high-performance liquid chromatograph –
a ProStar 410 autosampler equipped with a 0.5 mL sample loop (Varian, Palo Alto, California, USA), connected on-line with a liquid chromatograph (Varian, 240 pump) – and monitored by a 330 Varian PDA detector at the optimal wavelength for each component. Data processing was carried out using Star Chromatography Workstation version 5.52 software running on a personal computer. Characterisation of the spectra and the assessment of peak purity were performed by polyviw-2000 software.

Reagents
Ethanol (96% v/v) PA from Panreac (Barcelona, Spain), methanol (HPLC grade) and phosphoric acid (both supplied by Panreac), and n-hexane (LiChrosolv, Merck, Darmstadt, Germany), Folin–Ciocalteau reagent, sodium carbonate, gallic acid and AAPH (2,2′-azobis-2-methylpropanimidamide dihydrochloride) were from Sigma. All standards for the identification and quantitation of extract components were from Sigma–Aldrich (St. Louis, USA).

LITERATURE CITED


