Assessment of white grape pomace from winemaking as source of bioactive compounds, and its antiproliferative activity

M. José Jara-Palacios, Dolores Hernanz, Tania Cifuentes-Gomez, M. Luisa Escudero-Gilete, Francisco J. Heredia, Jeremy P.E. Spencer

Abstract

The antiproliferative effects of a purified white grape pomace extract (PWGPE), as well as of some phenolic standards on colon cancer cells were examined. The phenolic composition of the PWGPE was determined by rapid resolution liquid chromatography/mass spectrometry (RRLC/MS). The PWGPE had 92.6, 43.3 and 6.01 mg/g of flavanols, flavonols and phenolic acids, respectively and, along with pure catechin, epicatechin, quercetin and gallic acid, they were all found capable of inhibiting cellular proliferation. PWGPE (100 μg/ml) inhibited the proliferation of cells by 52.1% at 48 h, whilst catechin, epicatechin, quercetin and gallic acid (60 μg/ml) inhibited growth by 65.2%, 62.2%, 81.0% and 71.0%, respectively, at 72 h. The PWGPE is an interesting source of phenolic compounds with antiproliferative properties, that could be of interest in the food and pharmaceutical industries.

Keywords: Caco-2, Colorectal cancer, Grape pomace, Phenolic compounds

1. Introduction

Wine seems to be beneficial to health and, therefore, a moderate and regular consumption of wine is recommended. During winemaking, the polyphenols of the grape are transferred to the wine, but a high proportion still remains in the solid winemaking by-products. Grape pomace, consisting of the waste seeds, skins and stems resulting from the winemaking process, has been postulated to be a relatively good source of dietary bioactive compounds, such as polyphenols (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2010; Rodríguez Montealegre, Romero Peces, Chacón Vozmediano, Martínez Gascueña, & García Romero, 2006), with recent data reporting its antioxidant, anti-radical, antimicrobial, anti-inflammation and anticancer activities and its cardioprotective action (Rodríguez-Rodríguez et al., 2012; Sagdic et al., 2011; Xia, Deng, Guo, & Li, 2010). Therefore, the consumption of polyphenols has been associated with reduced risk of chronic diseases, including cardiovascular and neurodegenerative diseases, and cancer, e.g., colon cancer (Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010).

The importance of polyphenols makes the study of byproducts of interest for the food, nutraceutical, pharmaceutical and chemical industries. In recent years, there had been a growing interest in the use of byproducts rich in polyphenols emanating, from the winemaking industry, to produce extracts and novel products for the maintenance of human health (Kulkarni, De Santos, Kattamuri, Rossi, & Brewer, 2011; Perumalla & Hettiarachchy, 2011). As such, the extraction of polyphenols from waste material deriving from traditional industrial practices represents an attractive, sustainable and cost effective source of these high-value biological bioactives, which could be incorporated into foods. Due to the increasing demand for nutraceutical and antioxidant compounds, the study of grape pomace may be useful for industrial purposes.

Colorectal cancer is the third most prevalent cancer worldwide (Chan & Giovannucci, 2010) and there is intense interest in the development of novel chemopreventive products and/or the production of anti-cancer agents from novel, industrial waste-stream sources. Several studies have indicated the anti-cancer potential of polyphenolic compounds, and in particular their ability to inhibit the proliferation of cancer cells via their effects on the cell-cycle and/or apoptosis (Lee et al., 2006; Ramos, Rodríguez-Ramiro, Martín, Goya, & Bravo, 2011; Vu et al., 2012), and have suggested their use as novel dietary chemopreventive agents (Araújo, Gonçalves, & Martel, 2011). For example, the antiproliferative effects of polyphenolic extracts from olive oil (Corona et al., 2007), red wine (Gómez-Alonso et al., 2012), tomato (Saunders, 2009), araçá (Medina et al., 2011), raspberry (Coates et al., 2007), and...
and cranberry (Vu et al., 2012) have been reported. However, to date there are no previous data on the actions of grape pomace extracts, although their potential to protect against oxidative stress has been shown in Caenorhabditis elegans (Jara-Palacios et al., 2013). These phenolic extracts are a relatively rich source of bioactive compounds, which could be exploited in the food and pharmaceutical industries.

The current study aimed to address this by investigating the potential anti-cancer effects of a purified white grape pomace extract (PWGPE), rich in phenolic compounds, on colon adenocarcinoma cell growth, in relation to the individual effects of pure polyphenols relevant to PWGPE, notably epicatechin, catechin, quercetin and gallic acid.

2. Materials and methods

2.1. Reagents and standards

Methanol, n-hexane and hydrochloric acid were purchased from Labscan (Dublin, Ireland). Formic acid, HPLC-grade acetone, trisodium carbonate, and Folin–Ciocalteau reagent were obtained from Panreac (Barcelona, Spain). Gallic acid, protocatechuic acid, (+)-catechin, (−)-epicatechin, quercetin, kaempferol, quercetin-3-O-rutinoside (rutin), ferulic acid, caffeic acid, p-coumaric acid, and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (Madrid, Spain). Quercetin-3-O-glucoside and kaempferol-3-O-glucoside were obtained from Extrasynthese (Genay, France). Alamar Blue was from Abd Serotec (Kidlington, UK). Fehling’s reagents, dimethyl sulfoxide (DMSO), sulforhodamine B-based assay kit and all other reagents used for assay with cells were obtained from Sigma Aldrich (UK).

The stock solutions of phenolic standards were prepared in water at concentration of 100 mg/L. The corresponding dilutions were made up from the stock solutions to assess the antiproliferative effects.

2.2. Extraction of phenolic compounds from grape pomace

Grape pomace of the variety Zalema, D.O. “Condado de Huelva” (Spain), collected after winemaking, was supplied by “Viñolca del Condado” winery (Bollullos Par del Condado, Spain). Grape pomace was immediately frozen at −20°C, freeze-dried for 48 h and finally ground to a fine powder. The freeze-dried grape pomace (50 g) was extracted with 75% methanol as described previously (Jara-Palacios et al., 2014). An Agilent 1260 chromatographic system (Agilent Technologies, Palo Alto, CA, USA), equipped with a diode-array detector, and an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany), equipped with an ESI source, and a triple-quadrupole ion trap mass analyser were used. Phenolic compounds were identified by comparing their retention time, UV–vis spectra, and mass spectral data with authentic standards where available. The compounds were quantified using peak area data of resolved peaks at 280, 320 and 370 nm, for flavanols, phenolic acids and flavonols, respectively. Procyanidins were quantified, using catechin as the standard, whilst caftaric, fertaric and coutaric acids were quantified using caffeic, ferulic and p-coumaric acids, respectively. Quercetin and isorhamnetin derivatives were quantified as quercetin, and kaempferol derivates as kaempferol. Detailed retention times, UV–vis data and mass spectrometric data were shown previously in another paper (Jara-Palacios et al., 2014). The samples were analyzed in triplicate, and the results were expressed as mg of phenolic compound per g of PWGPE.

2.3. Determination of individual phenolic compounds: RRLC/MS analysis

Chromatographic analysis was carried out as previously described (Jara-Palacios et al., 2014). An Agilent 1260 chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a diode-array detector, and an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany), equipped with an ESI source, and a triple-quadrupole ion trap mass analyser were used. Phenolic compounds were identified by comparing their retention time, UV–vis spectra, and mass spectral data with authentic standards where available. The compounds were quantified using peak area data of resolved peaks at 280, 320 and 370 nm, for flavanols, phenolic acids and flavonols, respectively. Procyanidins were quantified, using catechin as the standard, whilst caftaric, fertaric and coutaric acids were quantified using caffeic, ferulic and p-coumaric acids, respectively. Quercetin and isorhamnetin derivatives were quantified as quercetin, and kaempferol derivates as kaempferol. Detailed retention times, UV–vis data and mass spectrometric data were shown previously in another paper (Jara-Palacios et al., 2014). The samples were analyzed in triplicate, and the results were expressed as mg of gallic acid per g of PWGPE.

2.4. Determination of total phenolic content

The total phenolic content (TPC) was determined by the Folin–Ciocalteu spectrophotometric method, as described previously (Jara-Palacios et al., 2014). Analyses were conducted in triplicate and gallic acid was employed as a calibration standard. Results were expressed as mg of gallic acid per g of PWGPE.

2.5. Cell culture

2.5.1. General

Human colon adenocarcinoma cells (Caco-2, ECACC Salisbury, Wiltshire, UK) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 20% heat-inactivated bovine serum, 2 mM l-glutamine, 1% non-essential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were seeded in 24-well plates at low confluence (2.5 × 10⁴ per well) and then exposed to the PWGPE (10, 25, 50 and 100 μg/ml), catechin (30 and 60 μg/ml), epicatechin (30 and 60 μg/ml), quercetin (30 and 60 μg/ml), gallic acid (30 and 60 μg/ml), or vehicle (DMSO 1%), 8 h after seeding.

2.5.2. Cellular proliferation and viability

The ability of the PWGPE to inhibit cellular proliferation was tested, using the sulforhodamine B (SRB) assay. Cells were harvested following 24, 48 and 72 h in culture and treated by the addition of 125 μl of ice-cold TCA (4°C; 1 h). After fixing, media were removed, cells were washed and total biomass was determined using SRB (250 μl of 0.4% SRB; 30 min). Unincorporated dye was removed by washing with 1% acetic acid, whilst cell incorporated dye was solubilised using Tris–base (10 mM). Dye incorporation, reflecting cell biomass, was measured at 492 nm, using a GENios microplate reader (TECAN, Reading, UK).

Viability was assessed after 24 h of treatment with PWGPE at different concentrations by the Alamar blue assay, as described previously (Vauzour, Vafeiadou, Rice-Evans, Williams, & Spencer, 2007). Briefly, Alamar Blue solution (10% v/v) was added to each well, and plates were incubated for 3 h, prior to fluorescence being measured at 540 nm excitation and 612 nm emission on the GENios microplate reader.
2.6. Statistical analysis

For the statistical treatment of the data, the Statistica v.8.0 software was used. One-way analysis of variance (ANOVA) was employed to establish if antiproliferative effects differed significantly between (a) the PWGPE and the phenolic standards and (b) those with the vehicle (DMSO 1%).

3. Results and discussion

3.1. Phenolic composition

The total phenolic content of the PWGPE was 365 mg/g (Table 1), 11 flavanols, 9 flavonols and 5 phenolic acids were identified and quantified by RRLC/MS, with flavanols being the major compounds detected accounting for 65% of total phenolic compounds (92.6 mg flavanols/g PWGPE), followed by flavonols (43.3 mg/g PWGPE, 31%) and phenolic acids (6.01 mg/g PWGPE, 4%) (Table 1). The most abundant flavanol was procyanidin B1 (15.5 mg/g PWGPE), followed by catechin, procyanidin B2-3-O-gallate, and procyanidin tetramer 1 (12.2, 11.8 and 10.8 mg/g PWGPE, respectively). The main flavonols were quercetin-3-O-glucoside and quercetin-3-O-glucuronide (16.9 and 15.8 mg/g PWGPE, respectively). The most abundant phenolic acid was gallic acid (3.03 mg/g PWGPE). These phenolic compounds were found in other extracts from Zalema grape pomace, but in different amounts (Jara-Palacios et al., 2013, 2014), which may be due to the extraction process.

3.2. Effect on cell proliferation

PWGPE, at different concentrations (10, 25, 50 and 100 μg/ml), induced dose-dependent inhibition of cancer cell proliferation following 24, 48 and 72 h of exposure (Fig. 1). A significant increase (p < 0.05) in growth inhibition treated cells was observed after all treatments, relative to vehicle, with the exception of PWGPE at 10 μg/ml and 72 h of exposure (p > 0.05), whilst the 100 μg/ml dose for 48 h induced the greatest degree of inhibition of proliferation (52% growth inhibition). Our data agree with previous cell data indicating that polyphenols are capable of inhibiting the proliferation of colon adenocarcinoma cells in a dose-dependent manner. Notably, the polyphenol rich foods, e.g., olive oil (100 μg/ml), red wine (100 μg/ml) and a tomato extract (2.5 mg/ml) inhibited cancer cell growth over a similar time frame (Corona et al., 2007; Gómez-Alonso et al., 2012; Saunders, 2009).

Previous studies have indicated that such activity may be mediated via the potential of polyphenols and their metabolites to induce cell cycle blockage in the G2/M phase in adenocarcinoma cells, perhaps through their effects on COX-2 activity (Shan, Wang, & Li, 2009; Yasui, Kim, Oyama, & Tanaka, 2009). On the other hand, these antiproliferative effects may be mediated, in part, by their direct cytotoxic action (Gómez-Alonso et al., 2012). With respect to the latter, our data suggest that cell viability at 24 h was significantly (p < 0.05) reduced at higher exposure concentrations (Fig. 2). Our data agree with previous observations that a polyphenol-rich red wine and araçá fruit extracts also reduce cell viability (Gómez-Alonso et al., 2012; Medina et al., 2011).

The assayed concentrations of polyphenols (10–100 μg/ml) in the culture medium could be associated with those present in some food sources, such as white wine, cocoa, black currant, or tea (Lamuela-Raventós, Romero-Pérez, Andrés-Lacueva, & Tornero, 2005; Recamales, Sayago, González-Miret, & Hernanz, 2006; Wang, Lu, Miao, Xie, & Yang, 2008; Williamson & Clifford, 2010). These amounts of polyphenols have shown antiproliferative effects in the cells; however, human health effects of these compounds depend on their bioavailability. A previous study suggests that the phenolic composition of a food source could affect the microbiota and its catabolic activity, inducing changes that could in turn affect the bioavailability and potential bioactivity of polyphenols producing metabolites which might be responsible for observed health effects in vivo (Cueva et al., 2013). Therefore, although in vitro assays provide information on bioactivity of polyphenols, it is essential to know their bioavailability by in vivo studies, which would be interesting to look at in future.

In order to elaborate the mediating bioactive components of the extract used, individual phenolic compounds, gallic acid, catechin, quercetin and epicatechin were also tested for their effects on cancer cell growth (Fig. 3). A significant inhibition (p < 0.05) of proliferation was observed for all compounds, relative to vehicle-

![Fig. 1. Growth inhibition induced by treatment with PWGPE at different concentrations. Caco-2 cells were exposed to the treatment for 24, 48 and 72 h before SRB assay was conducted. Each value represents mean (n = 12) ± SD. a = p < 0.001; b = p < 0.01; c = p < 0.05.](image-url)
treated cells, ranging from 17% to 81% for epicatechin (30 μg/ml) and quercetin (60 μg/ml), respectively (Fig. 3). Indeed, the antiproliferative effects of the PWGPE were comparable to effects of catechin, epicatechin, gallic acid and quercetin at different concentrations. Caco-2 cells were exposed to the treatment for 24, 48 and 72 h before SRB assay was conducted. Each value represents mean (n = 9) ± SD.

4. Conclusions

In summary, a PWGPE obtained from the winemaking process of time and storage conditions on the phenolic composition and colour of white wine.

Fig. 3. Growth inhibition induced by treatment with catechin, epicatechin, quercetin and gallic acid at different concentrations. Caco-2 cells were exposed to the treatment for 24, 48 and 72 h before SRB assay was conducted. Each value represents mean (n = 9) ± SD.

Fig. 2. Viable cells determined after treatment for 24 h with different concentrations of PWGPE. Caco-2 cells were exposed to the treatment for 24 h before Alamar blue assay was conducted. Each value represents mean (n = 12) ± SD. a = p < 0.001; b = p < 0.01; c = p < 0.05.

may be the mediating components. This study has described the inhibitory nature of dietary polyphenolic compounds on colon cancer cell growth, but there is no clear evidence that consumption of polyphenols prevents cancer; therefore future work is necessary to establish this.

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