Portuguese winemaking residues as a potential source of natural anti-adenoviral agents

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Abstract
To date there are no licensed systemic or topical treatments in Europe or the USA for adenovirus infections. In the present paper, we evaluate the effect of a polyphenol-based grape extract (NE) obtained from Portuguese white-winemaking by-products, and Resveratrol in pure form, on adenovirus type 5 infection. For this purpose, recombinant adenovirus vectors (Ad-5) and a human-derived cell line (293) were used as models. The NE and Resveratrol at the used concentrations do not induce cell cytotoxicity or direct virucidal activity; however, they reduce 4.5 and 6.5 log (TCID50/ml) on total infectious Ad-5 production, respectively. The capacity of Ad-5 replication upon removal of NE and Resveratrol after 24 h post infection was also evaluated. In contrast to Resveratrol, the highest evaluated NE concentration inhibits irreversibly the Ad-5 replication. These results provide useful information for the use of NE and Resveratrol as potential sources of promising natural antiviral agents on Ad-5 infection.

Keywords: Grape extract, Resveratrol, antiviral activity, adenovirus, antioxidants, phenolic compounds

Introduction
Adenoviruses are the cause of a large number of infectious diseases that affect multiple organs, commonly respiratory and gastrointestinal tracts, and the ocular surface (Horwitz 2001). More recently, largely because of molecular diagnostics, adenovirus infection has been associated with a number of acute and chronic diseases, such as pulmonary dysplasia (Couroucli et al. 2000), chronic airway obstruction (Russkanen et al. 1997), myocarditis and sudden infant perinatal death (Bajanowski et al. 1996). However, some population groups, like transplant recipients and patients with underlying immunodeficiency, are particularly susceptible to adenoviruses turning the infection being more common and, often, more severe (Munoz et al. 1998;
Kojaoghlanian et al. 2003). Thus, adenoviral infections have occurred frequently
and have been related with a high incidence of morbidity and mortality, increasing
the economic impact associated with these infections. Currently there are no
licensed systemic or topical treatments in Europe or the USA for adenoviral
infections, increasing the interest in the development of effective anti-adenoviral drugs
(Kinchington et al. 2005).

At the present time, there are at least 51 distinct human adenovirus serotypes
identified (Sarantis et al. 2004) and all of them appear to be structurally identical,
containing a linear and double-stranded DNA genome encapsulated in an icosahedra
protein shell (Shenk 2001).

As an alternative to conventional chemical agents for the development of an effective
treatment, a large number of phytochemicals have been recognized as a way to control
viral infections (Kalvatchev et al. 1997; Yamasaki et al. 1998; Abad et al. 1999a, 1999b,
2000). They include terpenoids, alkaloids, fiber and phenolic compounds. In the past
several years, a large number of scientific reports have described the properties of
phenolic compounds from numerous natural products. Special interest has been paid
to grape polyphenols, not only for their health-beneficial properties but also for their
sensory properties in wine (Pinelo et al. 2005).

Grape is one of the major fruit crops worldwide and its harvest is about 60 millions
tonnes per year (Schieber et al. 2001). About 80% of the harvest is utilized for winemaking
and the grape waste is 20% of the weight of processed grapes (Lafka et al. 2007).

Recently, grape seeds and winemaking extracts have been studied for their anti-
microbial activity and preservative properties (Jayaprakasha et al. 2003; Baydar et al.
2004; Serra et al. 2008) and Rhodes et al. (2006) reported anti-listerial activity for
grape juice, skin and seed extract of a Vitis vinifera variety. Trans-Resveratrol (3,5,4¢-
trihydroxystilbene) is a naturally occurring phenolic compound that can be found in
grapes and consequently in grape products such as wine (Sieman and Creasy 1992).
Several reports has shown that Resveratrol (RV) has a wide range of biological
activities, likewise strong anti-carcinogenesis effects (Delmas et al. 2006; Jang et al.
1997), inhibition of platelet aggregation and coagulation (Pace-Asciak et al.
1996), cardioprotective effects (Zern and Fernandez 2005), antibacterial activity
(Docherty et al. 2001), antiviral properties (Docherty and Fu 1999; Heredia and
Davis 2000; Evers and Wang 2004; Wang and Heredia 2004; Palamara et al.
2005; Docherty et al. 2006; Drago et al. 2008; Berardi et al. 2009), and anti-aging
agent in treating age-related human diseases (Howitz et al. 2003). Such diverse
biological effects of RV might be attributed to its powerful antioxidant properties
and its ability to mediate important cellular signaling pathways (Jang et al. 1997). In
this work, the capacity of a synthetic form of RV and of a natural extract (NE)
containing polyphenols made from white-winemaking by-products of a widely spread
Portuguese cultivar “Arinto” in inhibiting Ad-5 infection were evaluated and com-
pared. This study provides useful information on the utilization of these products,
namely NE, as natural antiviral agents on Ad-5 infection.

Materials and methods

Materials

Folin–Ciocalteu reagent was from Panreac (Barcelona, Spain). Gallic acid, RV (purity
> 99.8%; molecular weight, 228.25), cell proliferation reagent 3-(4,5-dimethylthiazolyl-2)-
2,5-diphenyltetrazolium bromide (MTT) and the antibiotic solution (10 g/l streptomycin and 10,000 U/ml penicillin) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Glucose was from Merck (Darmstadt, Germany) and Dulbecco’s modified Eagle’s medium (DMEM) medium, fetal bovine serum (FBS), L-glutamine 200 mM (100×) and trypsin–ethylenediamine tetraacetic acid solution were from GIBCO (Invitrogen, Glasgow, UK).

Natural extract

NE was obtained using generally recognized as safe solvents from white-winemaking residues (grape skins and seeds) of a widely spread cultivar from the western Portuguese region (“Arinto” variety). Briefly, white grape residues were roughly milled in a blender and extracted with 50:50 H2O/EtOH mixture (2 ml solvent/g residue) during 30 min and at room temperature using a propeller stirrer, four-bladed, IKA® RW 20 (Staufen, Germany). The liquid obtained was centrifuged at 9,056 × g at 20°C during 15 min and the volume reduced seven times by a rotary evaporator. The extract was passed through 0.2 μm filter before storage at –20°C.

Phenolic determination

The total polyphenol concentration of NE was quantified by the Folin–Ciocalteu method (Singleton and Rossi 1965). In brief, the appropriate diluted solutions of extracts were oxidized with Folin–Ciocalteu reagent and the reaction was neutralized with sodium carbonate. Absorbance of the samples was measured at 765 nm on a spectrophotometer (Genesys™ 10 UV from Thermo Finnigan (San Jose, CA, USA)) after 30 min and at 40°C. Gallic acid was used as standard, and the result was expressed as means of three replicates (μg gallic acid equivalents [GAE]/ml extract).

Resveratrol determination

Analyses by liquid chromatography (LC) were performed with Surveyor equipment from Thermo Finnigan (San Jose, CA, USA). The mass spectroscopy (MS) system was an LCQ ion trap mass spectrometer (Thermo Finnigan) equipped with an electrospray ionization or atmospheric pressure chemical ionization source. The LC–MS system was run by Xcalibur version 1.3 software (Thermo Finnigan–Surveyor). Electrospray ionization and atmospheric pressure chemical ionization were tested as ionization sources in negative and positive polarity modes.

The analytical column was a RP-18 (100 × 2.1 mm i.d., 5 μm particle size) from Thermo with a guard column of the same type. The samples were injected using a 20 μl loop; the separations were carried out with a flow rate of 250 μl/min. The column temperature was 35°C. The mobile phase used for Resveratrol identification consisted of a mixture of eluent A (formic acid 0.5%) and eluent B (acetonitrile). The analyses were performed using the following program of eluents: 0–4 min, from 0 until 15% eluent B; 4–20 min, from 15 until 40% eluent B; 20–30 min, from 40 until 55% eluent B. In the mass spectrometer experiments, the following conditions were used for the atmospheric pressure chemical ionization source in positive mode: vaporizer temperature, 470°C; discharge current, 5 μA; temperature of the heated capillary,
175°C. Nitrogen was used as the sheath gas and as the auxiliary gas with a gas flow rate of 80 and 20 arbitrary units, respectively. LC–MS was performed in the selected ion monitoring mode for m/z 229. For fragmentation, a collision energy of 37% was used with the scanning range of 60–2,000 amu.

The LC–MS quantification of trans-resveratrol in the sample was done by comparison with a trans-resveratrol standard with a concentration of 1 ppm (Figure 1).

Virus, cells and media

The utilized Ad-5 strain, containing the gene that codifies for green fluorescence protein, was produced in 293 cells and purified by cesium chloride gradient. Ad-5 was stored at −20°C in a Tris buffer formulation (Tris 10 mM pH 8, 2 mM MgCl₂ with 0.5 M trehalose).

Anchorage-dependent 293 cells, purchased from ATCC (ATCC-CRL-1573), were cultured in DMEM supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FBS, 4.5 g/l glucose and 1% (v/v) antibiotic solution (10 g/l streptomycin and 10,000 U/ml penicillin).

Cellular toxicity

Cytotoxicity of RV and NE was assessed by the MTT assay described in detail in previous work (Frade et al. 2007). Stock solutions of RV were prepared in 100% ethanol and diluted in culture medium DMEM with 0.5% FBS to the final concentrations ranging up to 250 µM. Controls were preformed with 0.5% ethanol, the maximum percentage present in the culture medium. NE solutions were prepared by dissolving the aqueous extract into DMEM with 0.5% FBS in a concentration range up to 80 µg/ml polyphenols.

The MTT assay measures the mitochondrial dehydrogenase activity of living cells that can be correlated with the cell number. 293 cells were plated in six-well tissue culture plates at a density of 1 × 10⁶ cells/well and incubated for 48 h with different concentrations of RV (0–250 µM) and NE (0–800 µg/ml polyphenols) in 0.5% FBS medium. After this period the colorimetric reagent MTT (0.5 g/l) was added to each well and left for 4 h. MTT is reduced to a purple formazan product by mitochondrial reductase enzymes active in viable cells, and therefore the amount of formazan product is proportional to the number of viable cells. The reaction was stopped with dimethyl-sulfoxide in each well and formazan was quantified by measurement of the absorbance at 540 nm in a plate reader. The ratio between the absorbance of NE or RV treated cells and the absorbance of non-treated cells (control) was used to determine the cell viability. The concentrations at which NE and RV induced 50% of inhibition of cell proliferation (CC₅₀) were estimated. Assays were performed in triplicate.

Antiviral assays

To determine the effect of RV and NE on Ad-5 replication, 293 cells were grown in six-well plates and infected with Ad-5 at 100 multiplicity of infection. After a 2 h period for viral adsorption, the culture medium was removed and replaced with fresh medium containing various concentrations of RV or NE, for a period of 46 h (corresponding to 48 h post infection [hpi]). At 2, 6, 24 and 48 hpi, samples of culture medium containing
Figure 1. Mass spectra of a standard solution of trans-resveratrol (RV) and natural grape extract (NE). (a) Selected ion monitoring mode at $m/z$ 229. (b) MS2 of precursor ion $m/z$ 229.
the released virus and the cells, containing the intracellular virus, were collected and frozen at \(-20^\circ C\). For viral quantification, the samples were thawed and titrated on 293 cells using 96-well plates by the end-point dilution assay and the results expressed as the median tissue culture infectious dose (TCID\(_{50}\)). The titer was calculated according to the method of Spearman and Kraber (Darling and Boose 1998).

In order to evaluate whether the observed RV inhibitory effect in Ad-5 replication is reversible, more experiments were performed with 50 and 75 \(\mu\)M RV. For this experimental approach, the culture medium containing RV was removed at 6, 12 and 24 hpi and replaced by fresh medium without RV. Samples were collected every 6 h and viruses were quantified.

The RV was dissolved in ethanol and diluted to the final concentrations in 0.5% FBS culture medium. The higher percentage of ethanol tested was 0.5% (v/v). NE concentrations were prepared by dissolving the aqueous extract into 0.5% FBS culture medium and the maximum concentration used was 800 \(\mu\)g GAE/ml. Control experiments were done with 0.5% FBS culture medium only and containing 0.5% ethanol (v/v).

Direct inactivation of Ad-5 virus replication

To verify whether RV or NE were capable of directly inactivating Ad-5 replication, a virus inoculum was incubated alone during 48 h, with control media, media containing 75 \(\mu\)M RV or media containing NE 800 \(\mu\)g GAE/ml. During this period, several aliquots were taken and the virus titrated by the endpoint dilution assay. The results are expressed as log (TCID\(_{50}\)/ml).

Statistical analysis

The data presented are the means ± standard deviation. Data significance was calculated according to \(t\)-tests. \(P<0.05\) was considered significant.

Results

Inhibition of virus replication

In this work, resveratrol and a natural grape extract were investigated for their potential antiviral activity against adenovirus type 5. NE is a complex mixture of phenolic compounds with total phenolic concentration of 3,400 \(\mu\)g GAE/mL and containing 1.5 \(\mu\)g/ml (6.6 \(\mu\)M) trans-Resveratrol (Figure 1).

The results demonstrated that RV clearly reduced Ad-5 replication in a dose-dependent manner (Figure 2). When infected cells were treated with the lowest concentration of RV (25 \(\mu\)M) during the 48 h, viral replication was not affected.

For 50 \(\mu\)M RV, at 24 hpi, the Ad-5 yield was already abrogated in 2.5 log. However, after 48 hpi, the effect of this concentration on the adenoviral replication was tenuous—only 1 log reduction was achieved. A total inhibition of viral replication was attained for the maximum concentration used—75 \(\mu\)M RV. Figure 2 shows a 6.5 log of inhibition at 48 hpi when 75 \(\mu\)M phenolic compound is added to infected cells. This value is higher than 1 log of inactivation, which is the yield reduction observed when the adenoviruses were directly incubated with RV during the same period of time (Figure 3).
NE was also able to inhibit Ad-5 replication in a dose-dependent manner. Figure 4 presents the total infectious Ad-5 particles obtained after 48 h with or without (control) permanent contact with different concentrations of NE and RV. The highest tested concentration of NE (800 mg GAE/ml with 1.6 μM trans-resveratrol) showed a decrease of 5 log (TCID50/ml) in total virus, which is only 1.3-fold lower than pure RV (75 μM).

Additionally, the effect of NE (800 mg GAE/ml) on direct inactivation of Ad5 was similar to 75 μM of RV (Figure 3).

Cytotoxicity was assayed for RV and NE using the MTT reagent according to the described method. The RV concentration causing CC50 was 150 μM. Moreover, 0.5% ethanol, which corresponds to the maximum concentration present in highest RV solutions, has no effect on 293 cells’ survival.

Among the NE concentration range assessed, CC50 for the 293 cells was never attained (data not shown). The highest NE concentration tested was 1,000 mg GAE/ml.

**Is the inhibition reversible?**

Data obtained show that Ad-5 was able to re-replicate if culture medium containing 75 μM RV was kept for a period up to 12 hpi, whereas this recovery was just partial if left for a longer period of 24 hpi, never attaining the viral replication kinetics of the control experiments (Figure 5). Similar experiments were performed with NE at the concentration of 800 μg GAE/mL, and the replacement of culture medium done only at 24 hpi.
Replication of Ad-5 at 48 hpi, for the highest concentrations tested (75 μM RV and 800 μg GAE/ml NE), with and without replacement of culture medium at 24 hpi, is presented in Figure 6. Results revealed that NE had an irreversible antiviral action against Ad-5 and virus yield was not affected by removal of NE at 24 hpi. The RV concentration in NE was about 47 times lower (1.6 μM) than highest RV tested concentration (75 μM).

Figure 3. Evaluation of direct inactivating effects of RV (75 μM) and NE (800 μg GAE/ml or 1.6 μM RV) on Ad-5 replication at 48 hpi. An inoculum of virus was incubated alone during 48 h, with control media, media containing 75 μM RV or media containing NE 800 μg GAE/ml. At the indicated time of exposure, several aliquots were removed and virus titrated by the end-point dilution assay. Data presented as means ± standard deviation. Statistically there was no significant inactivation of Ad-5 by RV or NE (P > 0.05) when compared with control.

Figure 4. Effect of RV and NE in Ad-5 replication at 48 hpi. A total of 293 cells were infected with Ad-5 and treated with different concentrations of RV and NE, and replication was allowed to occur for 48 h. Data presented as means ± standard deviation. Statistically there was significant inhibition of Ad-5 replication by RV (75 μM) and NE (1.6 μM RV) (P < 0.05) when compared with control.
Figure 5. Evaluation of the reversibility of RV antiviral effect. A total of 293 cells were infected with Ad-5 and incubated in control culture medium (▲) or culture medium containing 75 µM (★) RV. RV-containing media was not removed (a) or was removed and replaced by culture media without RV at 6 h (b), 12 h (c) and 24 h (d). Arrows indicate the time that the medium was removed and replaced. Data presented as means ± standard deviation.

Figure 6. Comparative study of antiviral activity of RV and NE with or without removal of culture media at 24 hpi. A total of 293 cells were infected with Ad-5 and treated with RV (75 µM) and NE (1.6 µM), and replication was allowed to occur for 48 h. Data presented as means ± standard deviation. Statistically there was no significant inhibition of Ad-5 replication by RV (75 µM) at 24 hpi (P > 0.05) when compared with control.
Discussion

The data attained demonstrate that to achieve an irreversible and efficient inhibition of Ad-5 replication, it is necessary to have resveratrol in permanent contact with the virus/cells at concentration of 75 μM. If RV is removed before 24 hpi, the replication of virus can proceed in what appeared to be a normal fashion approach. This result suggests that RV is more efficient during the early phase of Ad-5 replication. Accordingly, Evers and Wang (2004) demonstrated that RV inhibited human cytomegalovirus replication by prevention of appearance of immediate–early, early and late viral proteins. Moreover, Docherty et al. (2006) demonstrated that the mechanism by which RV inhibited varicella-zoster virus (VZV) replication in MRC-5 cells is related with the blocking/suppressing expression of IE62, an essential immediate–early viral protein. Herein, the concentration of RV required to completely inhibit VZV replication was found to be 219 μM and, in accordance with the results obtained in this work with Ad5, RV does not significantly inactive VZV directly.

RV was also reported to inhibit the synthesis of virus DNA in the infected cell in a study performed with polyomavirus (Berardi et al. 2009). The present work suggests that anti-adenoviral activity of RV manifests itself through several mechanisms into the host cell. In fact, the molecular mechanisms by which RV probably inhibits inflammation, coronary heart disease and cancer closely parallel a number of cellular signaling and stress activation events that are apparently required for productive adenovirus infection. RV is reported to inhibit the activation of a variety of stress induced, proinflammatory transcription factors, such as NF-κB, that are induced in adenovirus-infected cells (Brunder and Kovesdi 1997; Faith et al. 2006).

As suggested for RV, the NE activity was also manifested through several mechanisms into the host cell since the highest tested NE concentration (800 μg GAE/ml) was not capable of directly inactivating Ad-5 replication after 48 h of incubation (Figure 3). Therefore, the result obtained in the comparative study (Figure 6) suggests that the stronger NE antiviral action can be due to a positive synergetic effect of RV with other powerful antioxidant compounds and/or to the presence of other components in the natural extract with antiviral activity.

Data presented in this work are very encouraging for the development of effective natural anti-adenoviral agents. Further studies with NE are being developed in order to relate the antiviral action with a specific antioxidant (or group of) present in the extract. Furthermore, the understandings of the mechanisms involved are very important for the development of an effective natural treatment.

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References


