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The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV)

Vicente Micol [1], Nuria Caturla [1], Laura Pérez-Fons, Vicente Más, Luis Pérez, Amparo Estepa *

Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, E-03202-Elche, Alicante, Spain

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Abstract

A commercial plant extract derived from olive tree leaf (*Olea europaea*) (LExt) and its major compound, oleuropein (Ole), inhibited the in vitro infectivity of the viral haemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus. Incubation of virus with LExt or Ole before infection reduced the viral infectivity to 10 and 30%, respectively. Furthermore, LExt drastically decreased VHSV titers and viral protein accumulation (virucidal effect) in a dose dependent manner when added to cell monolayers 36 h post-infection. On the other hand, both the LExt and Ole were able to inhibit cell-to-cell membrane fusion induced by VHSV in uninfected cells, suggesting interactions with viral envelope. Therefore, we propose that *O. europaea* could be used as a potential source of promising natural antivirals, which have demonstrated to lack impact on health and environment. In addition, Ole could be used to design other related antiviral agents.

Keywords: Oleuropein; Olive leaf extract; VHSV; Antiviral; Viral membrane fusion

1. Introduction

Many screening efforts have been made to find antiviral agents from natural sources. Plants have long been used as remedies, and many are now being collected and examined in an attempt to identify possible sources of antivirals (Abad et al., 2000). In the last decades and as an alternative to conventional chemical agents, a large number of phytochemicals have been recognized as a way to control infections caused by viruses (Kalvatchev et al., 1997; Yamasaki et al., 1998; Abad et al., 1999a, 1999b, 2000). Leaves from olive tree, *Olea europaea*, are rich in biophenols (BPs), such as oleuropein (Ole) (Fig. 1), verbascoside, ligstroside, tyrosol or hydroxytyrosol. These compounds have shown several biological activities such as antioxidant (Visioli et al., 1998; Benavente-García et al., 2000) antithrombotic, and even skin photoprotective properties (Saija and Uccella, 2001). Furthermore, some of these compounds have demonstrated antimicrobial activity by inhibiting the growth of a wide variety of bacteria, fungi and viruses (Renis, 1969; Hirschman, 1972). The major BP of the olive leaf ethanolic extract is the secoiridoid oleuropein (varying from 20 to 25% (w/w) total dry weight) (Benavente-García et al., 2000) a glucoside with hydroxyaromatic functionality deriving from the shikimate and phenylpropanoid metabolism. Recently, Ole has been claimed in a U.S. patent to have potent antiviral activities against herpes mononucleosis, hepatitis virus, rotavirus, bovine rhinovirus, canine parvovirus, and feline leukaemia virus (Fredrickson, 2000). In addition, Ole has also exhibited a significant antiviral activity against respiratory syncytial virus and parainfluenza type 3 virus (Ma et al., 2001). To date, the mechanism of its antiviral activity is unknown.

In the search for safe and effective natural antiviral agents, we tested the antiviral activity of an olive extract (LExt), *O. europaea*, and its major component, the oleuropein, against a model rhabdovirus such as the viral haemorrhagic

* Corresponding author. Tel.: +34 96 6658436; fax: +34 96 6658758.
E-mail address: aestepa@umh.es (A. Estepa).
[1] Both authors contributed evenly.

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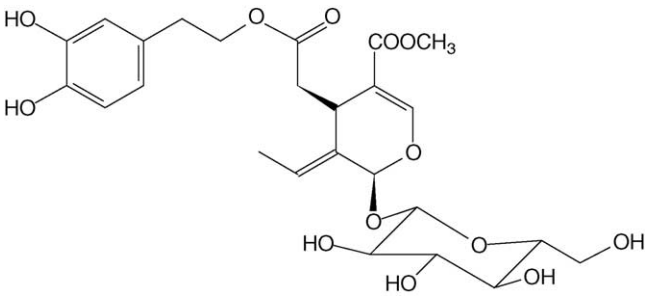

Fig. 1. Chemical structure of oleuropein, the major component of the olive leaf extract.

septicaemia virus (VHSV), which infects continental and sea farmed fish and a wide range of wild marine species in Europe, North America and Japan (Lorenzen et al., 1993; Leong and Fryer, 1993; Lorenzo et al., 1995; Snow and Cunningham, 2000; Isshik et al., 2001). In Europe the yearly economic losses due to VHSV are estimated at about 50 million euros. Moreover, the fact that this viral infection should be notifiable to the OIE (Office International des Epizooties) when appearing indicates the importance of VHSV rhabdovirus. The virus usually causes systemic disease and haemorrhagic lesions mainly among young fish, with mortality rates as high as 90% (Benmansour et al., 1997). Our interest in this fish-infecting virus is due to consumer related issues, such as accumulation of possibly toxic substances both in the food chain and in the environment.

LExt and Ole showed in vitro capacity to inhibit viral infectivity in a dose dependent manner when preincubated with the virus before infecting cells and also when administered post-infection. Since it has been previously reported that Ole interacts with phospholipid bilayers (Paiva-Martins et al., 2003; Saija et al., 1998), we studied the possible interference of LExt and Ole with the membrane fusion event in order to postulate if the mechanism for viral inactivation is related to viral-cell membrane fusion process. The results firmly indicate that the olive leaf extract, *O. europaea*, can be considered as a potential source of natural, selective, safe, low environmental impact and cost-effective antivirals, with relevant interest in aquaculture. Furthermore, Ole can be considered as a lead compound for the rational design of therapeutic agents for other rhabdovirus and/or enveloped virus.

2. Materials and methods

2.1. Reagents

Olive leaf powered extract (LExt) (*Oleurolive Plus*, commercial trade mark) was obtained from Monteloeder, S.L. (Elche, Spain). Oleuropein (Ole) (Fig. 1) was obtained from Extrasynthese (Genay, France) and further purified by reverse phase preparative high performance liquid chromatography.

2.2. EPC cell cultures and virus

The fish cell line "*Epithelioma papulosum cyprini*" (EPC) (Fijan et al., 1983) used in this work derived from a skin tumour of carp (*Cyprinus carpio* L.) and was purchased from the European collection of cell cultures (ECACC no. 93120820). VHSV 07.71 EPC cell monolayers were maintained as previously reported (Basurco and Coll, 1989). The virus used to infect the EPC cell monolayers at 14 °C was the viral haemorrhagic septicaemia virus 07.71 isolated in France from rainbow trout, *Oncorhynchus mykiss*. (LeBerre et al., 1977). Supernatants from VHSV-07.71 infected EPC cell monolayers were clarified by centrifugation at 1000 × *g* during 20 min and kept in aliquots at −70 °C. Viruses from clarified supernatants were concentrated to 10^{11} foci forming units (ffu) per ml by ultracentrifugation at 100,000 × *g* during 45 min (Basurco and Coll, 1989).

2.3. Cytotoxicity assays

The cytotoxic effects of LExt or Ole on EPC cell monolayers were determined by quantifying the EPC cell viability using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium] assay (Cell Titer 96; Promega, Mannheim, Germany). Cytotoxicity was examined following 2 (acute cytotoxicity) or 7 days (chronic cytotoxicity) of EPC cell monolayers exposure to different concentrations (from 1 to 1450 μg/ml) of LExt or Ole. CC_{50} values, defined as the concentration at which a 50% cytotoxic effect was observed after 7 days of cell treatment, were calculated as the compound concentration required to reduce the MTS signal to 50% of the untreated EPC cell monolayers. Average values and standard deviations from two different experiments each by triplicate were used to calculate the CC_{50} values.

2.4. Viral infectivity assays

To test the influence of pre-incubation of VHSV with LExt or Ole from *O. europaea* on VHSV infectivity, a previously developed immunostaining focus assay was used (Lorenzo et al., 1996; Perez et al., 2002; Mas et al., 2002). Briefly, LExt or Ole at different concentrations (up to 54 μg/ml) were incubated with VHSV during 2 h at 14 °C in RPMI-1640 cell culture medium supplemented with 2% foetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 500 μg/ml gentamicin and 25 μg/ml amphotericin B. After incubation, the mixtures containing VHSV (200 ffu/well) plus compounds were added to EPC cell monolayers, grown in 96 well plates, during 2 h at 14 °C. Alternatively, cells, which had been preincubated with the same concentrations of LExt or Ole, during 2 h at 14 °C, were infected with VHSV (200 ffu/well). Then, in both cases the infected EPC cell monolayers were washed, medium devoid of inhibitors added and the plates incubated during 24 h at 14 °C. After incubation (24 h), the EPC cell monolayers were fixed during 10 min in cold methanol

and air-dried. Monoclonal antibody (MAb) 2C9 directed towards the N protein of VHSV diluted 1000-fold in dilution buffer (0.24 mM merthiolate, 5 g/l Tween 20, 50 mg/l of phenol red in PBS pH 6.8) were added to the wells (100 μl/well) and incubated during 1 h at room temperature. After washing with distilled water, 100 μl of peroxidase-labelled rabbit anti-IgG mouse antibody (Ab) (Nordic, Tilburg, The Netherlands) were added per well, and incubation was continued during 30 min. After three washings by immersion in distilled water, 50 μl of 1 mg/ml per well of diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) in PBS containing H_2O_2 were added (Sanz and Coll, 1992; Lorenzo et al., 1996) and the reaction allowed to proceed until brown foci were detected with an inverted microscope. Once washed with water and air dried, brown foci (DAB-stained foci) of 15–20 brown or DAB-stained cells (DAB-stained single cells) were counted with an inverted microscope (Leica Ltd., Cambridge, UK) with a 10× ocular eye grid (Lorenzo et al., 1996). The results were expressed as the percentage of infectivity and calculated by the formula: (number of foci in the presence of compounds/total number of foci in the absence of compounds) × 100. The concentration at which an infectivity inhibition of 50% was observed, 50% inhibition concentration (IC_{50}), was defined as the concentration of compounds, which reduced the percentage of VHSV-induced foci by 50% with respect to untreated virus and expressed in μg/ml.

2.5. Viral titer determination from VHSV-infected EPC cell cultures treated with antiviral agents

In attempt to investigate the possible virucidal effect of the compounds, the growth of VHSV in VHSV-infected EPC cell cultures treated with LExt or Ole 36 h post-infection was analysed. EPC cells were grown on 24-well plates, infected with VHSV (10^{-3} ffu/well) and incubated during 36 h at 14 °C. Then, either LExt or Ole was added at different concentrations (0.6–1 mg/ml) and 2 days later the presence of infective virus in culture supernatants was determined by a methylcellulose plaque formation assay. Control experiments in which LExt or Ole were added simultaneously at the time of infection ($t = 0$) were performed.

2.6. VHSV assay by methylcellulose plaque formation

EPC cells were grown on 24-well plates to 100% confluence and then infected with serial dilutions of untreated virus or virus treated with LExt or Ole at various concentrations. One hour after virus adsorption at 14 °C, the medium was replaced by 0.5 ml of 0.75% methylcellulose (Sigma, St. Louis, MO, USA) in RPMI-1640 cell culture medium supplemented with 2% of FCS. Plates were incubated at 14 °C until plaque formation (5 days). After fixing and staining with a 0.1% crystal violet solution in formalin, the number of plaques was counted by eye inspection. The results were expressed as plaque forming units (pfu).

2.7. VHSV protein detection by western blotting in VHSV-infected EPC cell monolayers treated with LExt or Ole

EPC cell monolayers grown in 25 cm^2 culture flasks were infected repeat during 2 h at 14 °C with 10^3 pfu of VHSV per cell and after viral inoculum was removed, fresh medium was added. Thirty-six hours post infection, either LExt or Ole (0.8 mg/ml) were added and the infected cells incubated at 14 °C. At the sixth day post-infection, cells were frozen and defrozen, and supernatants clarified by centrifugation at 6000 × g during 45 min. Viruses from clarified supernatants were concentrated by ultracentrifugation as described (Basurco and Coll, 1989) and resuspended in electrophoresis buffer. SDS-15% polyacrylamide gels were loaded with 20 μl samples in buffer containing mercaptoethanol. To identify viral proteins both molecular weight markers (BioRad, Richmond, VI, USA) and purified VHSV were analysed simultaneously. Two parallel gels were run, one for Coomassie staining and the other one for western blotting. Gels were transferred during 3 h at 125 mA in 2.5 mM Tris, 9 mM glycine and 20% methanol to nitrocellulose membranes (Bio-Rad, Richmond, VI, USA). Membranes were blocked with 2% dried milk, 0.05% Tween-20 and 0.3% rabbit serum in PBS. Membranes were incubated with a polyclonal antibody against VHSV (Fernandez-Alonso et al., 1998) before incubation with a 500-fold diluted peroxidase-conjugated rabbit anti-IgG mouse antibody (Nordic, Tilburg, The Netherlands). Finally, the nitrocellulose membranes were immersed in 1 mg/ml 4-chloro-1-naphtol (Sigma, St. Louis, MO, USA), 0.015% H_2O_2 pH 7.5, washed and dried. Protein bands were densitometrized using the Scion Image 4.0.2 Software (www.scionorg.com). Peak areas for G, N and pM1 + pM2 proteins of VHSV were quantified and represented. VHSV concentrated from these cultures and used for the western blot assays was also tested for any residual infectivity by the methylcellulose plaque assay as previously described.

2.8. Fusion induced by addition of VHSV to uninfected EPC cell cultures

The low pH (pH 6) VHSV-induced cell-to-cell fusion in uninfected EPC cell cultures was estimated by a syncytia formation assay (Mas et al., 2002). VHSV (8×10^9 ffu/ml) was incubated with media, LExt at 54 μg/ml or Ole at 54 μg/ml in RPMI-1640 supplemented with 20 mM HEPES, 20 mM MES and 2% FCS at pH 7.4 for 2 h at 14 °C. VHSV (8×10^6 ffu/well), VHSV + LExt (8×10^6 ffu/well), VHSV + Ole (8×10^6 ffu/well), LExt or Ole (both at 54 μg/ml) were added to EPC cell monolayers on 96-well plates in a final volume of 100 μl per well. The fusion assay was performed as previously described (Mas et al., 2002). To measure the extent of fusion, cells were then fixed with cold methanol, dried and stained with Giemsa (5 mg/ml in phosphate saline buffer) and number of nuclei in syncytia of three or more nuclei per syncytia was counted among

10,000 nuclei per well The percentage of fusion was calculated by the formula, number of nuclei in syncytia/total number of nuclei × 100 (Estepa and Coll, 1997). The results were expressed as the percentage of fusion relative to the one induced by VHSV by the following formula, percentage of fusion induced by VHSV preincubated with LExt or Ole/percentage of fusion induced by VHSV × 100 (Mas et al., 2002). Alternatively, VHSV was added to EPC cell monolayers that had been preincubated with the same concentrations of LExt or Ole and after washing cells, fusion was induced as above.

3. Results

3.1. Low cytotoxicity of LExt and Ole

To explore the potential use of LExt or Ole as an antiviral agent, we first tested its possible in vitro acute and chronic toxicity. Neither LExt nor Ole showed any cytotoxic effects on EPC cell cultures at concentrations lower than 1 and 0.6 mg/ml, respectively. The cytotoxic concentration 50% values (CC50), i.e. the concentration corresponding to a 50% cytotoxic effect after 7 days of cell treatment, for LExt and Ole were of 1250 and 700 μg/ml, respectively (data not shown). Similar CC50 values for Ole have been found in different mammalian cell lines (Babich and Visioli, 2003).

3.2. Inhibition of VHSV infectivity by LExt and Ole

The inhibitory effect of LExt or Ole on VHSV infectivity, measured as the number of foci formation in EPC cell monolayers, is shown in Fig. 2. Both the LExt and Ole inhibited foci formation of VHSV in a dose dependent manner when preincubated with VHSV before the infection (Fig. 2A). Preincubation of VHSV with a concentration as low as 2 μg/ml of LExt or Ole reduced VHSV infectivity to about 68 and 50% of the untreated VHSV controls, respectively. The percentage of infectivity decreased steadily as the compound concentrations were increased up to a minimum value of ∼10% at 54 μg/ml of LExt. This reduction of the viral infectivity is clearly observed in the pictures showed in Fig. 2C–E where the infection foci were drastically reduced at 54 μg/ml Ole (Fig. 2E), and almost completely abolished at 54 μg/ml LExt

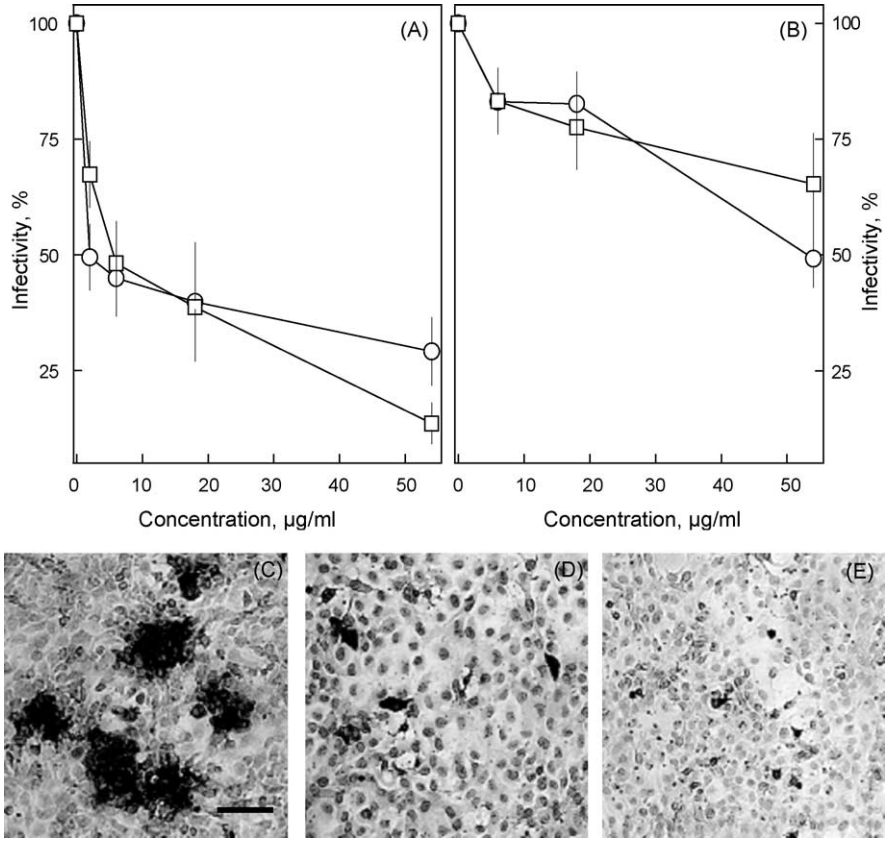

Fig. 2. Inhibition of VHSV infectivity by LExt and Ole. (A) VHSV was preincubated with LExt (*Oleurolive plus*) or Ole at indicated concentrations prior to infection of EPC cell monolayers. After infection time EPC cell monolayers were washed. (B) EPC monolayers were incubated with LExt or Ole, washed and then infected with VHSV. Foci of VHSV infected cells were quantified 24 h post-infection as indicated in Section 2. Results are presented as the percentage of infectivity and each point represents the mean of two independent experiments performed in triplicate. Error bars are standard deviations. LExt (open squares); Ole (open circles). Foci of infected cells from EPC cell cultures infected with VHSV (C), VHSV + 54 μg/ml Ole (D) and VHSV + 54 μg/ml LExt (E). Bar in panel C represents 100 μm.

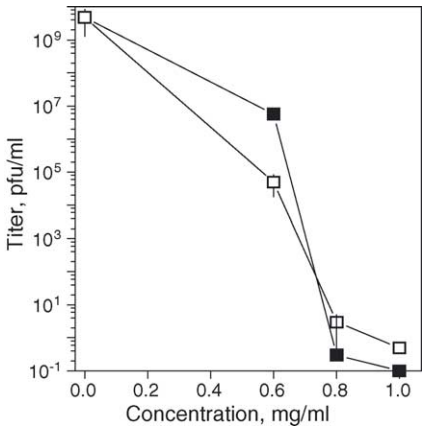

Fig. 3. LExt inhibits VHSV replication when added to VHSV-infected EPC cell monolayers. At 0 h and 36 h post-infection, LExt (*Oleurolive plus*) at the indicated concentrations was added to VHSV infected-EPC cells. Two days later, supernatants of infected cultures were harvested and the titer of recovered VHSV was estimated by the plaque assay. Each point represents the mean of two independent experiments performed in triplicate. Error bars are standard deviations. LExt added at 0 h post-infection (open squares); LExt added 36 h post-infection (filled squares).

(Fig. 2D), when compared to the untreated EPC cell monolayers (Fig. 2C). The 50% inhibitory concentration, IC_{50} value, concentration of compounds required to inhibit VHSV infectivity to 50%, and calculated for LExt and Ole were 5.89 and 1.98 μg/ml, respectively. In addition, when the EPC monolayers were preincubated with LExt or Ole at a concentration of 54 μg/ml (much lower than the concentration needed for cytotoxic effects) and then infected with intact virus, the VHSV infection was also reduced to 62 and 50%, respectively (Fig. 2B).

3.3. VHSV titer decreases in VHSV-infected EPC cell monolayers by post-infection treatment with LExt or Ole

In order to get some insight into the mechanism of action of LExt or Ole, we investigated whether the LExt or Ole could reduce VHSV titers when added after several rounds of VHSV replication in EPC cells. For that, EPC cells were infected with VHSV and 36 h later, LExt or Ole were added to the infected cell cultures (no compounds were added to control cultures). Two days after the addition of the compounds (3.5 days post-infection), VHSV titers were determined in the VHSV-infected cell culture supernatants by a plaque reduction assay. Fig. 3 shows the virus yield obtained in presence of 0.6, 0.8 and 1 mg/ml of LExt. In this experiment LExt and Ole concentrations were at least 1 × log higher than in Fig. 2 because now they are used to inhibit the higher viral loads resulting from a productive infection. In untreated VHSV-infected EPC cell cultures (control cultures), VHSV replicated normally and the viral titer recovered from supernatants of infected cultures was $>4 \times 10^9$ pfu/ml. In contrast, the viral titer recovered from infected EPC cells treated 36 h post-infection with LExt was significantly lower and decreased as the concentration of LExt was increased in a dose dependent manner (Fig. 3). At a concentration of 0.8 mg/ml, LExt almost completely inhibited the progression of the infection (less than 10^0 pfu/ml in culture supernatant) and at 1 mg/ml no virus was recovered (Fig. 3). The titer of VHSV recovered from VHSV-infected cultures treated with 0.8 mg/ml of Ole was about 10^4 pfu/ml (data not shown). However, we can not rule out that the lower titer could be due to some toxic effect of Ole at this concentration.

3.4. Viral protein synthesis decreases by post-infection treatment of VHSV-infected EPC cell cultures with LExt or Ole

To determine if the inhibition of VHSV titers caused by the treatment with LExt after the VHSV-cell infection was due to a decrease in the amount of virus particles released or a to loss of infectivity of the newly formed virus particles, the presence of VHSV proteins was analysed by western blotting. Six days post-infection, VHSV particles from supernatants of VHSV-infected EPC cell monolayers treated or not treated with LExt from 36 h post-infection were concentrated by ultracentrifugation. Then, viral proteins were analysed by immunoblot (Fig. 4, insert). Fig. 4 shows the densitometric quantitation of the bands corresponding to viral proteins. Although no differences were observed in the viral proteins composition, a nearly complete absence of viral proteins was observed in the EPC cell monolayers treated with LExt at 36 h post-infection. No residual infectivity was found in the virus concentrated from the supernatants used for western blot assays and deriving from VHSV-infected EPC cell monolayers treated with LExt (data not shown). These results indicate that VHSV replication is almost completely inhibited in VHSV-infected EPC cell monolayers treated with LExt. The results obtained using Ole as antiviral agents in similar assays were not included since the toxicity of Ole was close to the concentration needed to achieve a reduction of viral protein accumulation in this experiment.

3.5. VHSV-induced fusion in EPC cell monolayers is inhibited when VHSV is preincubated with LExt or Ole

Fusion of viral and cell membranes is a key step in rhabdovirus infection. To determine the influence of LExt or Ole on membrane fusion process, fusion was induced in cultures with VHSV treated or not treated with the LExt or Ole. When VHSV was preincubated with LExt or Ole before its addition to the EPC cell monolayers, the percentage of induced fusion dropped from 100 to 22% and 28% of the control, respectively. Neither LExt nor Ole induced significant percentages of fusion in the absence of VHSV (Fig. 5). In contrast, when fusion was induced with VHSV in EPC cell monolayers, previously treated with the same concentrations of the LExt or Ole, the fusion percentage values were similar to the controls (data not shown). This fact indicates that Ole interacts with the viral envelope producing VHSV particles with a reduced membrane-fusion capacity.

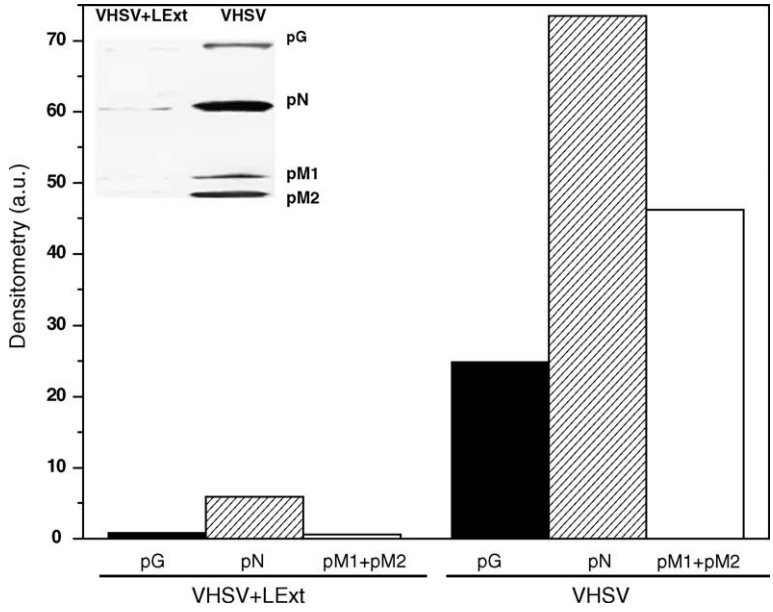

Fig. 4. Effect of LExt on VHSV protein accumulation in VHSV-infected EPC cell monolayers. EPC cell monolayers were infected with VHSV and 36 h later LExt was added. Six days after infection, cells and supernatants were harvested, concentrated by ultracentrifugation, and the presence of VHSV detected by western blot (insert). The blot is representative of three independent experiments. The relative amount of VHSV proteins was quantified by densitometric analysis of the western blots. VHSV, VHSV proteins in VHSV infected EPC cell monolayers; VHSV + LExt, VHSV proteins in VHSV-infected EPC cell monolayers treated with LExt 36 h post-infection. pG, VHSV-G glycoprotein; pN, VHSV-N nucleoprotein; pM1, VHSV-M1 phosphoprotein; pM2, VHSV-M2 matrix protein (a.u.), arbitrary units.

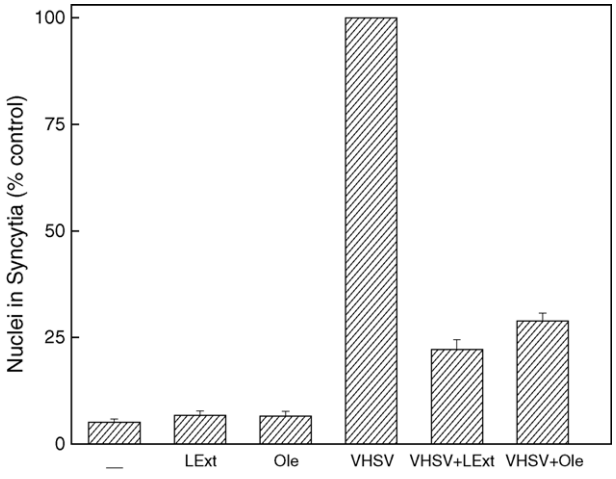

Fig. 5. Fusogenic activity of VHSV is reduced by pre-incubation of virus with LExt. Cell-to-cell fusion of uninfected EPC cell monolayers was induced as decribed in Section 2 by adding untreated VHSV, VHSV preincubated with LExt (*Oleurolive plus*) (VHSV + LExt), VHSV preincubated with Ole (VHSV + Ole), LExt (LExt) or Ole (Ole) to EPC cell monolayers. Fusion induced by untreated VHSV was considered as 100% value. *Y* axis represents total number of nuclei in syncytia larger than three cells. Each bar represents the mean of two independent experiments performed in triplicate. Error bars are standard deviations.

4. Discussion

Natural products are a relevant source of antiviral drugs (Donia and Hamann, 2003; Abad et al., 2000). For example, antiviral activities of phytochemicals have been described for vesicular stomatitis virus VSV (Eo et al., 1999; Eo et al., 2001; Chiba et al., 1992), an important mammalian rhabdovirus. Nevertheless, there are no therapeutic options available for VHSV-fish aquaculture so far. In contrast to many other enveloped viruses, no inhibitory peptides derived neither from the segments of its surface glycoproteins nor from the screening of combinatorial libraries (Mas et al., 2002) have been described for viruses belonging to the rhabdoviridae family, included VHSV. This deficiency of inhibitors must be due to the complexity of the mechanisms involved in the infectivity/fusion steps of rhabdoviruses compared to other enveloped viruses (Mas et al., 2002). Ole has been shown to possess a potent antiviral activity against other DNA and RNA viruses (Fredrickson, 2000; Ma et al., 2001). Due to the above-mentioned facts, and in order to search for safe and effective natural antiviral agents against VHSV, we examined the effect of a commercial LExt (*Oleurolive Plus*) and its major component Ole on VHSV infectivity.

The results presented here show the inhibitory action of both LExt and Ole against VHSV when the virus was incubated with the agents before infecting the cells (Fig. 2A), suggesting a direct inactivation effect of the VHSV infectivity by the compounds. However, pretreatment of the EPC cell monolayers with LExt or Ole prior to infection with untreated virus also inhibited infection although only partially (Fig. 2B). These results suggest that the compounds may interact with or persist locally on the EPC cell surfaces. A similar profile of antiviral activity has been found with the rabbit antimicrobial peptide, α-defensin 1 (NP-1), which protected cells in vitro from infection by herpes simplex virus type 2 (HSV-2) (Sinha et al., 2003).

In addition, the LExt could effectively reduce the progression of VHSV infection when added after several rounds of VHSV replication (Fig. 3). Concretely, LExt was able to abolish virus titer (Fig. 3) and viral protein accumulation (Fig. 4) in EPC cell cultures infected with VHSV when added 36 h after cell infection.

The therapeutic index ($TI = CC_{50}/IC_{50}$) found for Ole in this study was approximately 240, which indicates a higher in vitro antiviral activity than those values previously published for Ole and other secoiridoids from *Oleaceae* (Ma et al., 2001). LExt (*Oleurolive Plus*) showed a similar TI, i.e. 255. Then, pure Ole retained 77% (w/v) of the antiviral activity of the whole extract. However, considering that only 25% of the dry weight of the extract was Ole, the higher antiviral activity of LExt compared to pure Ole suggests the existence of another possible additive or a synergistic antiviral effect among Ole and other minor components present in the LExt. This probable additive or synergistic effect in the LExt is under investigation. Given that the production cost of the LExt is much lower than the cost of pure Ole, we might conclude that the whole extract is a more convenient antiviral agent than pure Ole for some applications, particularly in the fish farming industry where large amounts of the compound would be required.

Many studies have shown that Ole possesses a strong antioxidative activity (Edgecombe et al., 2000; Briante et al., 2001) but this property does not seem directly related to its antiviral effect (Ma et al., 2001). On the other hand, the interaction of Ole with the surface of phospholipid bilayers has previously been reported (Paiva-Martins et al., 2003). When VHSV was preincubated with the LExt or Ole, cell-to-cell fusion induced at low pH by VHSV in uninfected cells was reduced to 25–30% (Fig. 5), therefore an effect of the Ole on lipid/protein components present on the VHSV envelope and involved in membrane fusion could be possible. It has previously been published that the interaction of the G glycoprotein of rhabdoviruses with anionic phospholipid components of cellular membranes seems to be a necessary step for a successful viral fusion (Estepa et al., 2001; Gaudin et al., 1992; Estepa and Coll, 1996; Lenard, 1993). In this way, Ole might induce changes on the VHSV envelope, which could interfere the interaction of glycoprotein G to anionic phospholipid domains of membranes and therefore inhibit the early fusion steps.

These results indicate that *O. europaea* extracts can be considered a potential source of antiviral agents for aquaculture, which could lead to a new generation of chemicals with negligible environmental impact to be safely administered to the aquatic media.

Acknowledgments

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