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Virucidal activity of ginger essential oil against caprine alphaherpesvirus-1

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ABSTRACT

The emergence of alphaherpesvirus strains resistant to commonly used antiviral drugs has prompted the research for alternative, biologically active anti-herpetic agents. Essential oils (EOs) have shown anti-infective properties against human herpes simplex viruses (HSV-1 and -2). *Caprine alphaherpesvirus* 1 (CpHV-1) induces genital lesions in its natural host and it is regarded as a useful homologous animal model for the study of HSV-2 infection, chiefly for the assessment of antiviral drugs in *in vivo* studies. In the present study we evaluated the activity *in vitro* of ginger EO (GEO) against CpHV-1. GEO was found to be effective as virucide on cell-free virus, inactivating CpHV-1 up to 100%. The virucidal activity of GEO is likely accounted for by disruption of herpesvirus envelope and its associated structures which are necessary for virus adsorption and entry into host cells. On the opposite, GEO was not able to inhibit virus adsorption and/or replication, as treatment of cells before and after infection did not abolish virus infectivity. GEO could be suggested for topical applications in *in vivo* experiments using CpHV-1/goat model, since the lipophilic nature of EOs favours their adsorption through the cutaneous/mucosal barrier, either alone or in conjunction with other molecules. These findings open several perspectives in terms of therapeutic possibilities for a number of human and animal alphaherpesviruses.

1. Introduction

Alphaherpesviruses are large enveloped DNA viruses (family Herpesviridae, subfamily Alphaherpesvirinae) and are regarded as major human and animal pathogens. About 60–95% of the adult human population is infected by either herpes simplex virus 1 (HSV-1) or HSV-2 (Chayavichitsilp et al., 2009). HSV-2 is a sexually transmitted human pathogen that causes painful genital ulcers with negative repercussions on the sexual and reproductive performance. HSV-2 infects over 500 million people worldwide and causes 23 million new infections every year, with a sharp increase in the last years (Looker et al., 2008). After primary infection, HSV-2 establishes a lifelong latent infection in the lumbosacral sensory ganglia. Virus reactivation can be triggered by several stimuli including immunosuppression, stress and hormonal changes. Reactivation may occur without clinical signs or may trigger recurrent painful ulcerations of the genital mucosa, which, in turn, increase the risk of acquiring human immunodeficiency virus (Andrei et al., 2005; Carson et al., 2001). Besides the sexual transmission, HSV-2 is correlated with a serious risk of mother to child infection during birth (Chen et al., 2000).

Herpeviruses also cause significant economic losses in livestock animal industry (Patel and Didlick, 2008). Caprine alphaherpesvirus 1 (CpHV-1) infection in goats is associated with vulvo-vaginitis, reproductive failure, abortions and stillbirth (Roperto et al., 2000; Gonzalez et al., 2017). CpHV-1- has a strong biological similarity to HSV-2 for genital lesions that triggers. Both the viruses exhibit tropism for the genital apparatus, causing ulcerative-necrotic lesions on the vulvar rima, and induce latent infection in sacral ganglia (Tempesta et al., 1999).

This allowed hypothesizing the use of herpesvirus infection of goat as a model for the study of HSV-2 infection, chiefly for the assessment of antiviral drugs in *in vivo* studies (Tempesta et al., 2008). Previous experiments have demonstrated the validity of this animal model, using synthetic and natural molecules. These drugs were tested *in vivo* with different administration protocols (Tempesta et al., 2008; Camero et al., 2016).

Previous studies have described various animal models for HSV-1 and -2 infection, such as mice, rabbits, guinea pigs and rhesus macaques identifying variations in the patterns of acute and latent heterologous infections determined experimentally by HSVs with respect to what has

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been observed during natural infection in the human host (Laycock et al., 1991; Scriba and Tatzber, 1981; Kwant-Mitchell et al., 2009; Aravantinou et al., 2017).

Several molecules have been developed for treatment of HSV infections. Acyclovir and related nucleoside analogues (viral DNA polymerase inhibitors) have significantly increased treatment efficacy of HSV infections. However, the extensive use of nucleoside analogues drugs has triggered the onset of drug resistance, decreasing significantly treatment efficacy over the years (Stranska et al., 2005).

The emergence of virus strains resistant to commonly used anti-alphaherpesvirus drugs is a problem in clinical settings, particularly in immunocompromised patients. This has prompted the research for alternative anti-herpetic agents, with a wide range of efficacy, without serious adverse effects and able to circumvent potential mechanisms of virus resistance.

Essential oils (EOs) have long been known for their officinal properties. In plants, they function as antibacterial, antiviral, antifungal and insecticidal agents. However, they may also attract some insects that are vital for germination and reproduction of plants (Pollini et al., 2017). In addition, EOs have analgesic, sedative, anti-inflammatory, spasmolytic, anaesthetic (Pollini et al., 2017) and antioxidant properties (Teixeira et al., 2012). EOs are also used to add flavour and fragrance to foods, drinks, and other goods and act as preservatives for foods and drinks (Hyldgaard et al., 2012). Because of their properties (Llana-Ruiz-Cabello et al., 2015; Pollini et al., 2017), EOs are now gaining attention in the field of drug discovery and development. There are several studies in which EOs have been used against HSV-1 and HSV-2. The EO of *Melissa officinalis* can inhibit replication of HSV-2 (Allahverdiyev et al., 2004). The EO of citronella completely inhibits HSV-1 replication (Minami et al., 2003). *Mentha piperita* has high levels of virucidal activity against HSV-1, HSV-2 and against an aciclovir-resistant HSV-1 strain in viral suspension tests (Schuhmacher et al., 2003). The EO of the Australian tea tree and, to a lesser extent, the EO of eucalyptus, have shown antiviral activity against HSV-1 and 2 (Schnitzler et al., 2001). The EO of *Santolina insularis* has shown antiviral activity in toto against HSV-1 and HSV-2 *in vitro* (De Logu et al., 2000). Ginger (*Zingiber officinalis Rosc.*) EO (GEO) significantly inhibited infectivity of HSV-2 virus in RC-37 cells and halted plaque formation by 90% (Koch et al., 2008). Popular traditions attribute different virtues to ginger. Asian populations have used ginger for centuries against influence and cold, for its antiseptic and anti-inflammatory properties. In Europe, ginger is used for its digestive properties and for curative effects on the digestive tract. In the present study we analysed the activity of GEO against CpHV-1 *in vitro*.

2. Materials and methods

2.1. Analysis of GEO

The composition of commercially available GEO used in our experiments has been confirmed by hyphenated gas chromatography with mass spectrometry (GC/MS) technique (Rosato et al., 2018a, 2018b).

Commercially available pure *Zingiber officinalis Rosc*. EO (Lot 140/0000324, 10.2018, 10 mL) was provided by Specchiasol (Bussolengo, Verona, - Italy). C7-C30 alkanes mixture and solvents, all of analytical grade, were purchased from Sigma Aldrich S.r.l. (Milan, Italy), filters were supplied by Agilent Technologies Italia S.p.a. (Milan, Italy). Gas chromatographic analyses were performed on HP GC/MS 6890N-5973 N MSD HP ChemStation equipped with autosampler and HP-5MS column (crosslinked 5% PH ME siloxane) 30 m × 0.25 mm × 0.25 µm Film Thickness. The following temperature program was applied: 40 °C (4 min), 4 °C per minute heating up to 280 °C (30 min). The mass spectrometer was performed in EI mode at 70 eV; the ion source temperature was 220 °C. The mass spectra were measured in the range of 35–360 amu (unified atomic mass unit). For chemical characterization, a standard solution of 100 µl of the pure EO in 1 ml of ethyl acetate was prepared. The solution was filtered and 1 µl was analysed by GC/MS. The sample was analysed in triplicate. Qualitative analysis was executed comparing the calculated Linear Retention Indices (LRI) and Similarity Index Mass Spectra (SI/MS) for the obtained peaks with the analogous data from NIST2011 and Adams 4th ed. (2007) databases (Adams, 2007) LRI of each compound was obtained by temperature programming analysis and was determined in relation to an homologous series of n-alkanes (C7–C30) under the same operating conditions. LRI was calculated following the Van den Dool and Kratz equation and compared with the Arithmetic Index (AI) from NIST2011 database (Adams, 2007). SI/MS were determined as reported by Koo et al. (2013). Semi-quantification of essential oil components was made by peak area normalization considering the same GC response of the detector towards all volatile constituents (Rosato et al., 2018a, 2018b).

2.2. Cells and virus

Madin Darby bovine kidney (MDBK) cells were cultured at 37 °C in a 5% CO2 atmosphere in Dulbecco-MEM supplemented with 10% foetal bovine serum, 100 IU / ml penicillin, 0,1 mg/ml streptomycin and 2 mM L-glutamine. The same medium was used for the antiviral assays. The CpHV-1 strain Ba-1 was cultured and titrated in MDBK cells. The virus stock with a titre of $10^{4.50}$ Tissue Culture Infectious Dose – $TCID_{50}$/50 µl was stored at −80 °C and used for the experiments.

2.3. Cytotoxicity assay

GEO cytotoxicity was assessed using the in vitro Toxicology Assay Kit (Sigma–Aldrich Srl, Milan, Italy), based on 3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyl tetrazolium bromide (XTT). The test was performed as described previously (Lanave et al., 2017), following the manufacturer's instructions. Confluent 24-h monolayers of MDBK cells grown in 96-well plates were used to assess the cytotoxicity of GEO at different concentrations (6.98, 13.96, 27.92, 55.84, 111.68 and 223.36 µg/ml). In all experiments, untreated cells and cells treated with equivalent dilutions of DMSO without GEO were used as control and vehicle control, respectively. The percentage of cytotoxicity was calculated using the formula: % Cytotoxicity = [(OD of control cells − OD of treated cells) × 100] / OD of control cells.

The cytotoxic concentration (CC_{20}) was defined as the concentration at which viability of the treated MDBK cells decreased to 20% with respect to the control cells.

On the basis of the results, the maximum non-cytotoxic concentration of the GEO was assessed and used for antiviral tests. The experiments were performed in triplicate.

2.4. Antiviral activity assay

On the basis of the cytotoxicity assay results, the antiviral activity against the CpHV-1 strain Ba-1 was evaluated using GEO at 55.84 µg/ml, below the cytotoxic threshold. To identify the step of viral inhibition by GEO against CpHV-1, two different protocols (A and B) were carried out. All the experiments were performed in triplicate.

2.4.1. Protocol A: virus infection of cell monolayers before treatment with GEO

Confluent monolayers of MDBK cells of 24 h in 24-well plates were used. The cells were infected with 100 µl of CpHV-1 containing 100 $TCID_{50}$. After virus adsorption for 1 h at 37 °C, the inoculum was removed, cell monolayers were washed once with D-MEM and GEO at 55.84 µg/ml was added. In untreated infected cells, D-MEM or equivalent dilution of DMSO without GEO were used to replace the inoculum and used as virus control and vehicle control, respectively. After 72 h, aliquots of the supernatants were collected for subsequent viral titration and for DNA quantification.

2.4.2. Protocol B: virus infection of cell monolayers after treatment with GEO

Confluent monolayers of MDBK cells of 24 h in 24-well plates were used. The cells were treated with GEO (1 ml) at 55.84 µg/ml for 1 h at 37 °C. After removing the medium, the monolayers were washed once with D-MEM and infected with 100 µl of CpHV-1 containing 100 $TCID_{50}$. After virus adsorption for 1 h at 37 °C, the inoculum was removed and the monolayers were washed with D-MEM before adding 1 ml of maintenance medium (D-MEM).

In untreated infected cells, D-MEM or equivalent dilution of DMSO without GEO were used to replace the inoculum and used as virus control and vehicle control, respectively. After 72 h, aliquots of each supernatants were collected for subsequent viral titration and for DNA quantification.

2.5. Virucidal activity assay

The potential inhibitory effect of GEO against CpHV-1 was evaluated by pre-treatment of the virus (100 and 1000 $TCID_{50}$) with GEO at different concentrations starting from 55.84 µg/ml and exceeding the cytotoxic threshold (139.6 and 1396 µg/ml). In detail, 100 µl of CpHV-1 were treated with GEO (1 ml) at different concentrations at room temperature. After 4 and 8 h, the different mixtures of virus-GEO were subject to viral titration in MDBK cells.

2.6. Viral titration

Ten-fold dilutions (up to 10^{-8}) of each supernatants were titrated in quadruplicates in 96-well plates containing MDBK cells. The plates were incubated for 72 h at 37 °C in 5% CO_2. On the basis of cytopathic effect, the titer was calculated.

2.7. CpHV-1 DNA quantification

Aliquots of each supernatants of cell monolayers in the 24-well plates were tested by a quantitative PCR (qPCR) specific for CpHV-1, as previously described (Elia et al., 2008).

Briefly, tenfold dilutions of the CpHV-1 gC standard DNA, representing 10° to 10^{8} copies of DNA/10 µl of template, were made out in TE (Tris–HCl, EDTA) buffer containing 30 µg carrier RNA (tRNA from Escherichia coli, Sigma–Aldrich S.r.l., Milan, Italy) per ml. Aliquots of each dilution were frozen at −80 °C and used only once.

Two hundreds µl of supernatant were extracted using QIAamp Cador Pathogen Mini kit (Qiagen, S.p.A., Milan, Italy) according to the manufacturer's instructions.

Duplicates of CpHV-1 standards and DNA templates were subjected simultaneously to qPCR analysis. Amplification was carried out in a 25-µl reaction volume containing 12.5 µl of IQ™ Supermix (Bio-Rad Laboratories S.r.l.), 900 nM of each primer (CpHV-1 For 5′-TACCTCT TTCCCGCGCCCACG-3′ and CpHV-1 Rev 5′-TGTACACGCCCTCGGTC GCC-3′), 200 nM of probe CpHV-1Pb (5′-FAM − CCGCCTGCCCCTCAC CATCCGCTCC-TAMRA-3′) (Elia et al., 2008) and 10 µl of DNA. The thermal cycle protocol included activation of iTaq DNA polymerase at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 1 min and primer annealing and extension at 70 °C for 1 min.

2.8. Data analysis

After logarithmic conversion of GEO concentrations, the data obtained in the cytotoxicity assays were analysed by a non-linear curve fitting procedure. Goodness of fit was tested by non-linear regression analysis of the dose-response curve (GraphPad Prism v3 program Intuitive Software for Science, San Diego, CA, USA).

From the fitted dose–response curves obtained in each experiment, the non-cytotoxic concentration (CC_{20}) was considered as the compound concentration at which viability of treated MDBK cells decreased by no more than 20% with respect to the negative control. Data from GC/MS analyses were expressed as area % ± SEM.

Data from antiviral assays were expressed as mean ± SD. Shapiro-Wilk test was used to assess the normality of distribution. Data were analysed by T-Student test for independent samples or one-way Analysis of Variance (ANOVA) for repeated measures using Tukey test as post hoc test (statistical significance set at 0.05). Statistical analyses were performed with the software MedCalc Statistical Software version 16.4.3 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2016).

Table 1
Chemical composition of GEO. In bold the predominant components.

PK#	Area% ± SEM	Library/ID	LRI	AI	SI/MS
1	0.15 ± 0.013	Hexanal	805	801	91
2	1.73 ± 0.12	Alpha-Pinene	935	932	97
3	**4.74** ± 0.27	**Camphene**	947	946	96
4	0.71 ± 0.11	Beta-Pinene	976	974	97
5	0.41 ± 0.074	Prenylacetone	987	986	90
6	**4.35** ± 0.57	**Alpha-Phellandrene**	1003	1002	87
7	1.93 ± 0.42	Eucalyptol	1025	1026	98
8	0.17 ± 0.023	Terpinolene	1087	1086	96
9	0.23 ± 0.091	Linalol	1094	1095	91
10	1.01 ± 0.12	Endo Borneol	1164	1165	97
11	0.52 ± 0.24	Alpha-Terpineol	1187	1186	91
12	0.13 ± 0.065	Decanal	1206	1201	87
13	0.13 ± 0.061	Citronellol	1228	1223	96
14	0.25 ± 0.098	Geraniol	1252	1249	90
15	0.13 ± 0.096	Bornyl acetate	1284	1287	98
16	0.22 ± 0.10	2-Undecanone	1294	1293	94
17	0.72 ± 0.13	Alpha-Cubebene	1348	1345	95
18	0.58 ± 0.21	Cyclosativene	1364	1362	94
19	0.17 ± 0.096	2-Butyl-2-octenal	1375	1372	96
20	0.49 ± 0.17	Geranyl Acetate	1385	1379	91
21	0.14 ± 0.098	Caryophyllene	1425	1417	93
22	0.17 ± 0.065	Alpha-Bergamotene	1430	1432	93
23	0.56 ± 0.19	Gamma-Elemene	1439	1434	98
24	0.73 ± 0.13	Beta-Farnesene	1451	1454	97
25	0.56 ± 0.15	Alloaromadendrene	1457	1458	92
26	0.21 ± 0.096	Alpha-elemene	1467	1469	90
27	**15.2** ± 1.12	**Alpha-Curcumene**	1479	1481	99
28	**32.1** ± 1.78	**Zingiberene**	1495	1493	93
29	0.29 ± 0.11	Alpha-Muurolene	1502	1500	97
30	**7.23** ± 0.98	**Alpha-Farnesene**	1506	1505	93
31	2.29 ± 0.25	Beta-Bisabolene	1507	1505	93
32	**10.91** ± 1.10	**Beta-Sesquiphellandrene**	1523	1521	90
33	1.27 ± 0.27	Cubenene	1529	1533	93
34	0.41 ± 0.23	cis-Sesquisabinene hydrate	1539	1542	86
35	0.51 ± 0.12	Beta-Elemol	1542	1548	91
36	0.87 ± 0.23	Beta-Nerolidol	1562	1561	90
37	0.39 ± 0.29	trans-Sesquisabinene hydrate	1579	1577	93
38	0.25 ± 0.11	Beta-Eudesmol	1653	1649	99
	92.85 ± 2.81				

PK#: Compounds are listed in order of their elution from a HP-5MS column. LRI: Linear Retention Index on HP-5MS column, experimentally determined using homologous series of C7-C30 alkanes. AI: Arithmetic Index (Adams. 2007); SI/MS: Similarity Index/Mass Spectrum (NIST. 2011 Database).

3. Results

3.1. Analytical details of GEO

A total of 105 components were identified in GEO, with 38 components corresponding to 92.85% of the mixture. Zingiberene was the predominant component, accounting for 32% of the mixture. The composition of the EO has been summarized in Table 1.

3.2. Cytotoxicity

The cytotoxicity of GEO was determined by microscopic examination of cell morphology and measurement of cell viability by the XTT

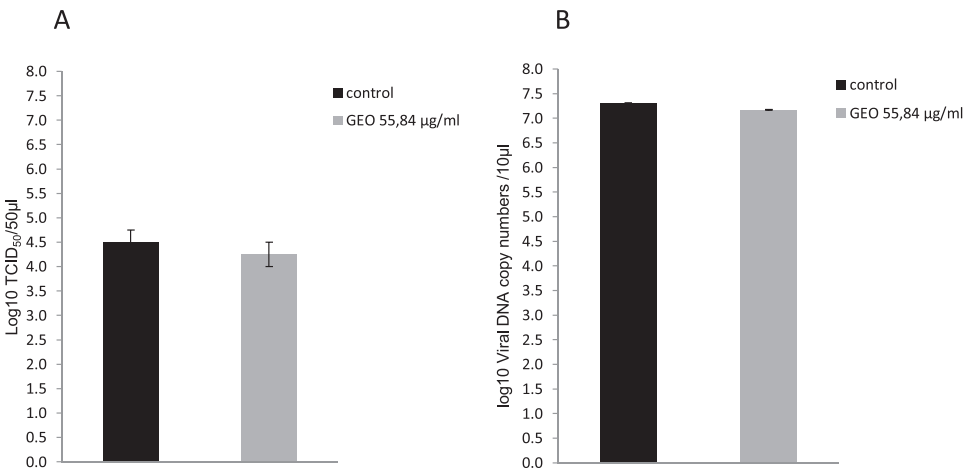

Fig. 1. Virus infection of cell monolayers before treatment with GEO. Antiviral effect of GEO (55.84 µg/ml) against CpHV-1. MDBK cells were infected with virus before the treatment with GEO before the infection. Supernatants from untreated and treated MDBK cells were collected at 72 h post infection and titrated on cells. (A) Viral titres were evaluated by endpoint dilution method and expressed as log10 $TCID_{50}$/50 µl. (B) Viral nucleic acids were quantified by qPCR and expressed as log10 viral DNA copy numbers/10 µl. Bars in the figures indicate the means. Error bars indicate the standard deviation.

colorimetric method after exposing the cells to various concentrations of the compound (6.98, 13.96, 27.92, 55.84, 111.68, 223.36 µg/ml) for 72 h. The intensity and variety of the cellular morphological changes (loss of cell monolayer, granulation, cytoplasmic vacuolization, stretching and narrowing of cell extensions and darkening of the cell borders) were dose-dependent (Lanave et al., 2017). Cytotoxicity was assessed by measuring the absorbance signal spectrophotometrically. In all the experiments, DMSO did not show any effect on cells.

On the basis of fitted dose–response curves, CC_{20} value of GEO was assessed at 75.28 µg/ml.

3.3. Antiviral activity

3.3.1. Protocol A: virus infection of cell monolayers before treatment with GEO

By comparing the viral titre of untreated infected cells (mean = 4.50 log10 $TCID_{50}$/50 µl, SD = 0.25 log10 $TCID_{50}$/50 µl) with infected cells treated with GEO (mean = 4.25 log10 $TCID_{50}$/50 µl, SD = 0.25 log10 $TCID_{50}$/50 µl), a slight average decrease of the viral titre (0.25 log10) was induced by GEO although without statistical significance ($p > 0.05$) (Fig. 1A). Comparing log10 viral DNA copies/10 µl of untreated infected cells (mean = 7.30 log10 DNA viral copy numbers/10 µl, SD = 0.02 log10 DNA viral copy numbers/10 µl) with infected cells treated with GEO (mean = 7.17 log10 DNA viral copy numbers/10 µl, SD = 0.02 log10 DNA viral copy numbers/10 µl) an average decrease of 0.13 log10 DNA viral copy numbers/10 µl was determined although without statistical significance ($p > 0.05$) (Fig. 1B). In the untreated infected cells with equivalent dilutions of DMSO without GEO, viral titres and viral DNA loads were not inhibited by DMSO compared to virus control (data not shown).

3.3.2. Protocol B: virus infection of cell monolayers after treatment with GEO

When comparing the viral titre of the untreated infected cells (mean = 4.50 log10 $TCID_{50}$/50 µl, SD = 0.25 log10 $TCID_{50}$/50 µl) with infected cells treated with GEO (mean = 4.25 log10 $TCID_{50}$/50 µl, SD = 0.25 log10 $TCID_{50}$/50 µl), GEO induced a slight average decrease of the viral titre (0.25 log10), although without any statistical significance ($p > 0.05$) (Fig. 2A). When assessing viral DNA load in untreated infected cells (mean = 7.33 log10 DNA viral copy numbers/10 µl, SD = 0.02 log10 DNA viral copy numbers/10 µl) and infected cells treated with GEO (mean = 7.16 log10 DNA viral copy numbers/10 µl, SD = 0.01 log10 DNA viral copy numbers/10 µl), GEO determined an average decrease of 0.17 log10 DNA viral copy numbers /10 µl without any statistical significance ($p > 0.05$) (Fig. 2B). In the untreated infected cells with equivalent dilutions of DMSO without GEO, viral titres and viral DNA loads were not inhibited by DMSO compared to virus control (data not shown).

3.4. Virucidal activity

The virucidal effect of GEO at different concentrations (55.84, 139.6 and 1396 µg/ml) was evaluated against CpHV-1 using 100 and 1000 $TCID_{50}$. The virus was treated with GEO for 4 and 8 h at room temperature.

CpHV-1 (100 $TCID_{50}$) treated for 4 h with GEO at 55.84, 139.6 and 1396 µg/ml showed significant reduction of viral titre of 0.75 log10 $TCID_{50}$/50 µl (33.3%, $p < 0.05$), 1.75 log10 (77.8%, $p < 0.01$) and 2.25 log10 (100%, $p < 0.01$), respectively, compared to the virus control (2.25 log10) (Fig. 3A). After 8 h GEO at 55.84 µg/ml determined significant decrease of viral titre of 1.25 log10 (71.4%. $p < 0.01$) compared to the virus control (1.75 log10) whilst GEO at 139.6 and 1396 µg/ml inactivated completely CpHV-1 (100%, $p < 0.01$) (Fig. 3B). In the ANOVA model, the results of viral titration of CpHV-1 (100 $TCID_{50}$/50 µl) treated with GEO at different concentrations (55.84, 139.6 and 1396 µg/ml) was compared with the virus control, showing a statistically significant effect ($F = 65$, $p < 0.001$). GEO at 55.84 µg/ml used against CpHV-1 (1000 $TCID_{50}$/50 µl) after 4 h did not decrease viral titre (0%, $p > 0.05$) compared to the virus control (3.00 log10). GEO at 139.6 and 1396 µg/ml determined a reduction of viral titre as high as 1.50 log10 (50%, $p < 0.01$) and 3.00 log10 (100%, $p < 0.01$) respectively, compared to the virus control (3.00 log10) (Fig. 3C). After 8 h, GEO at 55.84 µg/ml determined a significant decrease of viral titre of 0.75 log10 (70%, $p < 0.01$) compared to the virus control (2.50 log10) whilst GEO at 139.6 and 1396 µg/ml inactivated completely CpHV-1 (100%, $p < 0.01$) (Fig. 3D). When analysing with the ANOVA model the results of viral titration (1000 $TCID_{50}$) treated with GEO at different concentrations (55.84, 139.6 and 1396 µg/ml) compared with virus control, a statistically significant effect was observed ($F = 132$, $p < 0.001$).

4. Discussion

In recent years, many research groups have focused their attention on the anti-infective properties of natural substances. The antiviral activity of EOs has been investigated against various viruses (Pollini et al., 2017) including alphaherpesviruses (Allahverdiyev et al., 2004; Koch et al., 2008; Schnitzler et al., 2001; De Logu et al., 2000; Schuhmacher et al., 2003).

In this study we tested the anti-viral properties of GEO against CpHV-1 in vitro. The maximum non-cytotoxic dose of GEO on MDBK cells was as high as 75.28 µg/ml. Experiments to assess the antiviral activity against CpHV-1 were carried out using GEO at 55.84 µg/ml, i.e.

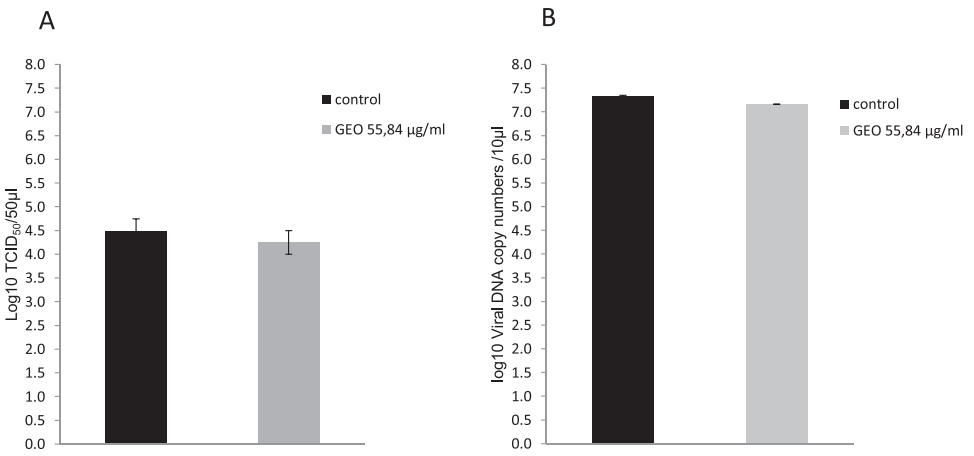

Fig. 2. Virus infection of cell monolayers after treatment with GEO. MDBK cells were pre-treated with GEO (55.84 µg/ml) before the infection with CpHV-1. Supernatants from cells untreated and treated-with-GEO were collected at 72 h post infection. (A) Viral titres were evaluated by endpoint dilution method and expressed as log10 $TCID_{50}$/50 µl. (B) Viral nucleic acids were quantified by qPCR and expressed as log10 viral DNA copy numbers/10 µl. Bars in the figures indicate the means. Error bars indicate the standard deviation.

slightly below the cytotoxic threshold, and cell monolayers were infected before and after treatment with the compound. In these experiments, we did not observe any statistically significant reduction of viral titer, suggesting that GEO is not able to inhibit virus adsorption and/or replication.

We also performed experiments to assess the virucidal activity of GEO which was found to be effective on cell-free virus. GEO was tested at the concentration of 55.84 µg/ml. Also, GEO was tested at concentrations exceeding the cytotoxic threshold (139.6 and 1396 µg/ml), since, if used as virucide, the molecule is not posed into direct contact with the cells. The activity of GEO was assessed using different virus doses (100 and 1000 $TCID_{50}$). Viral inactivation occurred in a dose-dependent fashion, reaching 100% when GEO was used at the highest concentration (1396 µg/ml), regardless of the viral doses and time intervals. Complete viral inactivation (100%) also occurred after 8 h when the compound was used at 139.6 µg/ml (Fig. 3). Significant decrease of CpHV-1 titres was observed in all the experiments except when CpHV-1 at 1000 $TCID_{50}$ was treated with GEO at 55.84 µg/ml for 4 h. However, increasing to 8 h the treatment caused a statistically significant reduction of virus titre. This suggests that time of contact is an important factor for virus inactivation.

The antiviral activity of GEO might rely on the fact that this substance is able to disrupt herpesvirus envelope and its associated structures which are necessary for adsorption and entry into host cells, as previously observed with other essential oils against HSV-1 (Schnitzler et al., 2007). Electron microscopy demonstrated disaggregation of HSV-1 envelope after pre-treatment with EOs (Shogan et al., 2006).

Assessing more in detail the virucidal effects of this vegetal extract

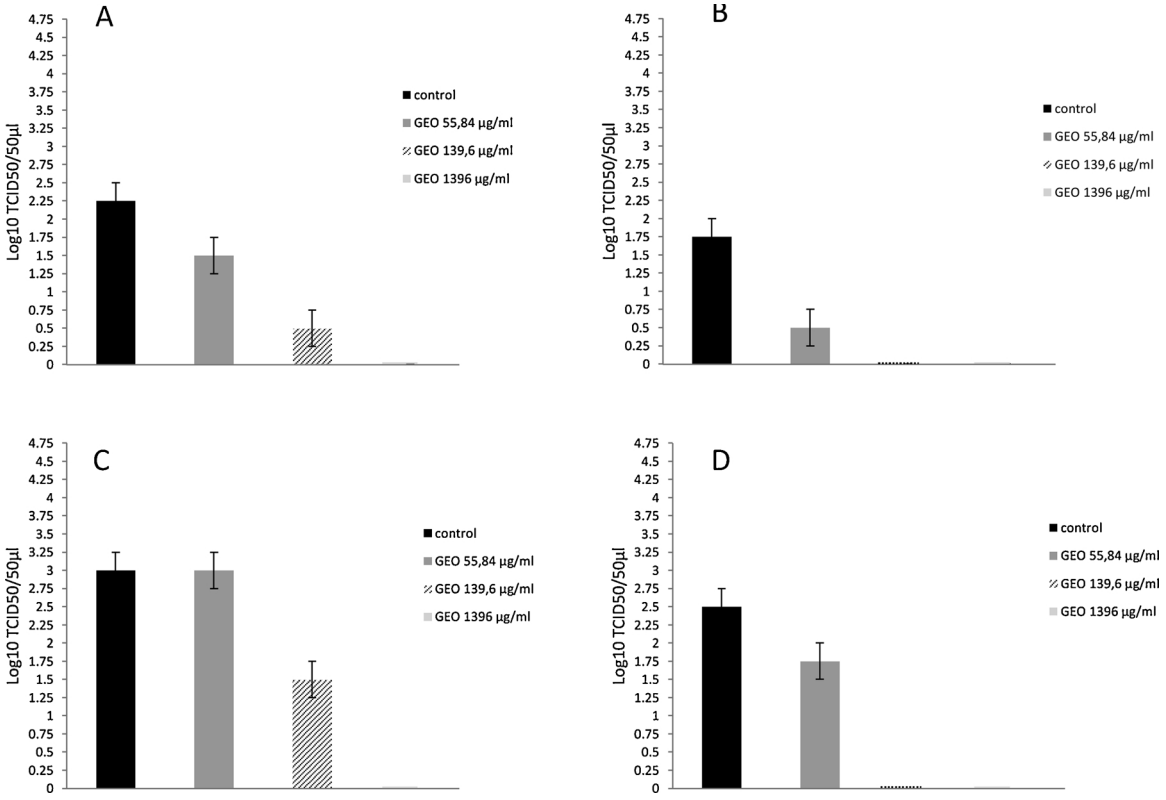

Fig. 3. Virucidal activity of GEO. Virucidal effect of GEO at different concentrations (55.84, 139.6 and 1396 µg/ml) against CpHV-1 (100 and 1000 $TCID_{50}$). The virus was incubated with GEO for 4 and 8 h at room temperature and subsequently titrated in MDBK cells. Viral titres of CpHV-1 (100 $TCID_{50}$) were evaluated after 4 h (A) and 8 h (B) of incubation and expressed as log10 $TCID_{50}$/50 µl. Viral titres of CpHV-1 (1000 $TCID_{50}$) were evaluated after 4 h (C) and 8 h (D) of incubation and expressed as log10 $TCID_{50}$/50 µl. Bars in the figures indicate the means. Error bars indicate the standard deviation.

when used as disinfectant or food additive could be of interest. In addition, GEO could be indicated for topical applications in *in vivo* experiments on CpHV-1 since their lipophilic nature enables EOs to penetrate the skin and block transmission of the virus at the mucosal surface, as observed for HSV-2 (Koch et al., 2008). Likewise, tea tree EO gel applied for the treatment of recurrent HSV-1 seems to be a therapeutic treatment option (Carson et al., 2001). On the opposite, systemic administration of EOs is considered highly toxic with possible side effects (Carson et al., 2001).

To date, the use of EOs in medical practice is limited. In the literature, there are only few data *in vivo* about the cytotoxicity of EOs, the mechanism of tissues penetration and the tolerated EO concentration. Currently, low concentrations of EOs or of their compounds are used as additives to ointments, creams, balms, lotions, gels, drops, or other specifics formulations applied externally in skin diseases, aromatherapy, and massages (Piątkowska and Rusiecka-Ziolkowska, 2016).

The chemical composition of GEO was dissected using gas-chromatographic analysis. Out of 38 distinct molecules, the main fractions were zingiribene, alpha-curcumene, beta-sesquiphellandrene, alpha-farnesene, camphene and alpha-phellandrene. In order to reduce the cytotoxicity of GEO, it could be interesting to identify the effective molecules and test them individually.

Synergistic effects have been observed when EOs are used together with various anti-infective substances (Piątkowska and Rusiecka-Ziolkowska, 2016; Rosato et al., 2018a) and this has been observed even in terms of antiviral activity (Chou, 2006). The synergic effects triggered by combination of more substances might allow using lower dosages of each molecule, thus reducing the toxic effects in prolonged therapies. Whether this also applies to GEO when used with other antiviral compounds should be assessed.

The process used for obtaining EOs is not expensive and therefore EOs have the potential for being be used as phyto-pharmaceuticals to reduce the costs of medical treatment. These findings open several perspectives in terms of therapeutic possibilities for a number of human and animal alphaherpesviruses and for the development of prophylaxis tools for disinfection and sanitation.

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