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In vitro inhibition of herpes simplex virus type 1 replication by *Mentha suaveolens* essential oil and its main component piperitenone oxide

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ABSTRACT

Several essential oils exert *in vitro* activity against bacteria and viruses and, among these latter, herpes simplex virus type 1 (HSV-1) is known to develop resistance to commonly used antiviral agents. Thus, the effects of the essential oil derived from *Mentha suaveolens* (EOMS) and its active principle piperitenone oxide (PEO) were tested in *in vitro* experimental model of infection with HSV-1. The 50% inhibitory concentration (IC_{50}) was determined at 5.1 μg/ml and 1.4 μg/ml for EOMS and PEO, respectively. Australian tea tree oil (TTO) was used as control, revealing an IC_{50} of 13.2 μg/ml. Moreover, a synergistic action against HSV-1 was observed when each oil was added in combination with acyclovir. In order to find out the mechanism of action, EOMS, PEO and TTO were added to the cells at different times during the virus life-cycle. Results obtained by yield reduction assay indicated that the antiviral activity of both compounds was principally due to an effect after viral adsorption. Indeed, no reduction of virus yield was observed when cells were treated during viral adsorption or pre-treated before viral infection. In particular, PEO exerted a strong inhibitory effect by interfering with a late step of HSV-1 life-cycle. HSV-1 infection is known to induce a pro-oxidative state with depletion of the main intracellular antioxidant glutathione and this redox change in the cell is important for viral replication. Interestingly, the treatment with PEO corrected this deficit, thus suggesting that the compound could interfere with some redox-sensitive cellular pathways exploited for viral replication. Overall our data suggest that both EOMS and PEO could be considered good candidates for novel anti-HSV-1 strategies, and need further exploration to better characterize the targets underlying their inhibition.

Introduction

Herpes simplex viruses (HSV) are ubiquitous agents causing a variety of diseases ranging in severity from mild to severe, and in certain cases, these may even become life threatenings, especially in immunocompromised patients (Christophers et al., 1998; Stránská et al., 2005; Ziyaeyan et al., 2007). In particular, HSV type 1 (HSV-1) is an important pathogen for humans. After primary infection, HSV-1 establishes a state of latency and persists for the lifetime of the host. In response to certain stimuli, the virus reactivates from latency and it may cause few recurrences in most people, although some patients experience frequent recurrent infections (De Chiara et al., 2012). A very effective treatment for HSV is available since the introduction of acyclovir (ACV), which is still the most commonly used chemotherapy (Brady and Bernstein, 2004). This antiviral agent can be used to shorten the course of the illness and decrease the severity of the clinical symptoms and may suppress the virus itself, by interfering with viral DNA polymerization through obligatory chain termination and competitive inhibition (Whitley and Roizman, 2001; Stránská et al., 2005). However, the widespread use of nucleoside based drugs has led to the emergence of drug-resistance HSV strains especially among immunocompromised patients (Kimberlin et al., 1995). Thus, the discovery of novel effective antiherpetic drugs without contraindications is of great interest.

In this field a great interest in substances of natural origin as therapeutic alternative has raised. In the last years essential oils

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(EOs) extracted from aromatic plants have been found to show antioxidant, anticancer, antimicrobial and other pharmacological activities (Holley and Dhaval, 2005; Basile et al., 2006; Naeini et al., 2009). A number of EOs has been also tested for antiviral activity and some of them have been shown to be potential antiviral agents. In particular, antiherpetic activities of some essential oils as Australian tea tree oil (Schnitzler et al., 2001; Reichling et al., 2006), peppermint oil (Schuhmacher et al., 2003), manuka oil (Reichling et al., 2005) and Sandalwood oil (Benencia and Courrèges, 1999) have previously been reported. The EOs show a complex chemical composition based on a number of constituents with low molecular weight, and their biological activities are due to a main component of the mixture, usually a monoterpene, or to combination of their multiple compounds or to synergism when added in combination with well-known antimicrobial agents (Schelz et al., 2006; Wagner and Ulrich-Merzenich, 2009). Plants of *Mentha suaveolens* Ehrh, collected in various regions of Morocco contain a high percentage of oxides such as piperitenone oxide (PEO), piperitone oxide (PO), terpenic alcohol and terpenic-5-ketones, all of which account for 65–90% of the total essential oil. Other plants of *Mentha* spp., like *Mentha rotundifolia*, contain PEO and PO (Fujita and Nezu, 1985; Lorenzo et al., 2002) even if in minor concentration. Note that *M. rotundifolia* is accepted as a hybrid of *M. longifolia* × *M. suaveolens* (Turker and Nuczi, 2007). The antimicrobial activity of PO, even if generally comparable to that of PEO, seems to be 2-fold lower than that of PEO against yeast (Hüsnü Can Baser et al., 2006). A recent study have addressed the *in vitro* candidastatic and candidacidal activity of the essential oil derived from *Mentha suaveolens* (EOMS) and its ability to accelerate the *in vivo* clearance of *Candida albicans* during vaginal infection (Pietrella et al., 2011). Since nothing is known about EOMS antiviral activity, we decided to investigate the potential role of this compound against HSV-1. In this study, we demonstrate that EOMS, as well as its main component PEO, carry out their stronger effect when added post infection. In particular, PEO exerts its antiviral activity by inhibiting a late step of HSV-1 life-cycle. Moreover, when EOMS or PEO were pre-incubated with the virus before infection, they showed a significant virucidal activity, thus interfering directly with the viral envelope.

Materials and methods

Essential oil purification

Essential oil of *Mentha suaveolens* (EOMS) was obtained from wild-type plants grown in Tarquinia forests (Rome, Italy) and extracted by 4-h hydro distillation of the leaves using a Clevenger-type apparatus as previously described (Angiolella et al., 2010), then analyzed for chemical composition by gas chromatography. One μl of the extract was diluted in 1 ml of CH_3OH and analyzed by GC–MS and GC-FID by using a turbomass Clarus 500 GC–MS/GC-FID from Perkin Elmer instruments. The following column and gas–chromatography conditions were employed: 60 m long, 0.25 mm i.d. Restek Stabilwax fused-silica capillary column with 1 ml min^{-1} helium internal flux, operated for 5 min at 60 °C then heated to 220 °C at a rate of 5 °C min^{-1}. The extraction products were identified by comparison of their mass spectra with those reported in the NIST and NBS libraries. Relative abundances of the components were derived by using the same instrumentation with the FID detector configuration. The GC-FID analysis revealed that piperitenone oxide (PEO) (Fig. 1) constitutes from 80% to 90% of EOMS (Fig. 2). Limonene, α-cubebene and pulegone were also present, among other minor constituents. PEO was purified by consecutive column chromatography (CC) eluting with $CHCl_3$/*n*-hexane (1:1) on silica gel 60. After three CC, the maximum purity of 97.2% was obtained monitoring the PEO purity by the above GC–MS protocol. Attempts to further purify PEO did not lead to any higher percentage.

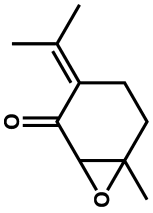

Fig. 1. The chemical structure of piperitenone oxide (PEO), the main constituent of EOMS.

Cell culture, virus production and infection

African green monkey kidney (Vero) cells were grown in RPMI 1640 medium (Gibco, Invitrogen Corporation, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Invitrogen Corporation, CA). Viability of cells was estimated by Trypan blue (0.02% final concentration) exclusion assay (Invitrogen Corporation). For virus production monolayers of VERO cells in 75-cm^2 tissue culture flasks were infected with HSV-1 strain F at a multiplicity of infection (m.o.i.) of 0.01. After 48 h at 37 °C, infected cells were harvested with 3 freeze-and-thaw cycles, and cellular debris were removed with low-speed centrifugation, and virus titer was measured by standard plaque assay (Killington and Powell, 1991). The titer of the virus preparation was 5×10^8 plaque forming units (pfu)/ml. The virus was stored at −70 °C until used.

Cellular toxicity

Cellular toxicity of EOMS, PEO and Australian tea tree oil (TTO) (Talia herbal, Italy) was tested *in vitro* according to a cell viability assay previously reported (Denizot and Lang, 1986; Mosmann, 1983). Monolayers of Vero cells were incubated with EOMS, PEO or TTO at concentrations of 1–500 μg/ml in RPMI 1640 for 24 h and the medium replaced with 50 μl of a 1 mg/ml solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma–Aldrich (St. Louis, MO)] in RPMI without phenol red (Sigma–Aldrich). Cells were incubated at 37 °C for 3 h, and 100 μl of acid-isopropanol (HCl 0.1 N in isopropanol) was added to each well. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read using an automatic

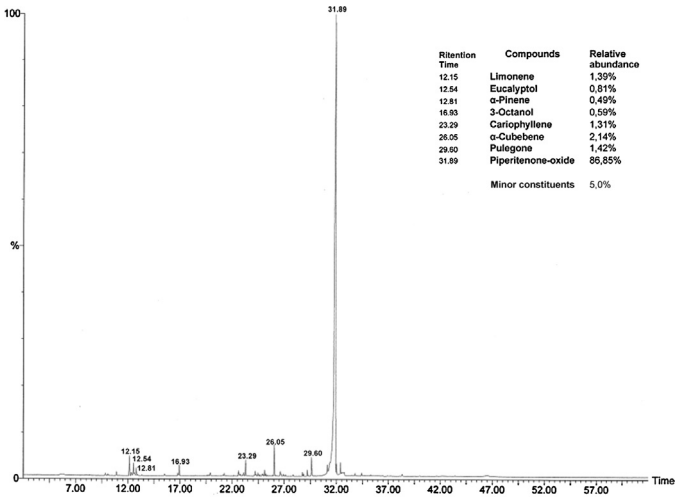

Fig. 2. GC–MS profile of *Mentha suaveolens* extract. GC analyses were performed using a turbomass Clarus 500 GC–MS/GC-FID from Perkin Elmer instruments.