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In vitro and *in vivo* Activity of Eugenol on Human Herpesvirus

F. Benencia[1]* and M.C. Courrèges[2]

[1]Departamento Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Piso 4, Ciudad Universitaria, (1428) Buenos Aires, Argentina
[2]Centro de Patología Experimental, Facultad de Medicina, Universidad de Buenos Aires, Uriburu 950. 5to. Piso, (1115) Buenos Aires, Argentina

Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) was tested for antiviral activity against HSV-1 and HSV-2 viruses. *In vitro*, it was found that the replication of these viruses was inhibited in the presence of this compound. Inhibitory concentration 50% values for the anti-HSV effects of eugenol were 25.6 μg/mL and 16.2 μg/mL for HSV-1 and HSV-2 respectively, 250 μg/mL being the maximum dose at which cytotoxicity was tested. Eugenol was virucidal and showed no cytotoxicity at the concentrations tested. Eugenol–acyclovir combinations synergistically inhibited herpesvirus replication *in vitro*. Topical application of eugenol delayed the development of herpesvirus induced keratitis in the mouse model. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: eugenol; antiviral activity; herpesvirus; herpetic keratitis; acyclovir.

INTRODUCTION

Due to the rapid emergence of drug resistant virus strains, the development of effective therapies for human viral diseases is dependent upon the identification of novel therapeutic agents with low toxicity. It is well known that plants have been used in medicine for thousands of years; in particular traditional medicines employing natural products have been shown to contain antiviral compounds *in vitro* (Locher *et al.*, 1996). In different countries screenings have been done in the search for antiviral activities in native medicinal plants (Kurokawa *et al.*, 1993; Pacheco *et al.*, 1993; Kurokawa *et al.*, 1995; Aruoma *et al.*, 1996; Locher *et al.*, 1996). Certain plant extracts inhibit virus replication and inactivate extracellular viruses. As examples, inhibition of replication of herpes simplex virus type 1 (HSV-1) by *Geranium sanguineum* L, influenza and human immunodeficiency virus by pine cone antitumour substance, murine cytomegalovirus by *Chlorella vulgaris*, poliovirus and HSV-1 by *Ulex europaeus* and HSV, pseudorabies virus, encephalomyocarditis virus and human immunodeficiency virus by *Opuntia streptacantha* extracts have been reported (Zgorniak-Nowosielska *et al.*, 1989; Nagata *et al.*, 1990; De Rodriguez *et al.*, 1990; Sagakimi *et al.*, 1991; Ahmad *et al.*, 1996). In addition, a number of plant compounds will neutralize virus infectivity; for example, neutralization of HSV, cytomegalovirus, Epstein–Barr virus and human immunodeficiency disease virus infectivity by polysaccharides and lectins (Ito and Barron, 1974; Ito *et al.*, 1978; Lifson *et al.*, 1986; Tabba *et al.*, 1989; Benencia *et al.*, 1997), inhibition of herpesvirus by tannins and related compounds, solanaceae glycalkaloids and saponins (Fukuchi *et al.*, 1989; Thorne *et al.*, 1985), anti-HIV activities of tetragalloylquinic acids and lignin derivatives (Suzuki *et al.*, 1989). Thus traditional medicines appear to be useful sources to search for new antiviral agents. Essential oils obtained from seeds, stem bark and roots of many plants have been widely used in traditional medicine. Among others, antibacterial, antifungal, immunomodulatory, antiinflammatory and antirheumatic activities have been described (Moran *et al.*, 1989; Phadke and Kulkarni, 1989; Garg and Siddiqui, 1992; Upadhayay *et al.*, 1992; Fu, 1993; Shapiro *et al.*, 1994; Agarwal, 1996; Nenoff *et al.*, 1996; Singh *et al.*, 1996; Al-Zuhair *et al.*, 1996). Alkenylbenzenes are an important group of naturally occurring food flavourings in herbs and spices. Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) a phenolic non-nutrient compound is the main component of oil of cloves and is also present in the essential oils of many plants including cinnamon, basil and nutmeg. Many studies have reported biological activities of clove oil and eugenol including therapeutic effects in dentistry (Woody and Davis, 1992; Markowitz *et al.*, 1992), antimicrobial activities (Saeki *et al.*, 1989; Briozzo *et al.*, 1989; Didry *et al.*, 1994), inhibitory effects on lipid peroxidation (Nagababu and Lakshmaiah, 1992) and peripheral vasodilatory effects (Sticht and Smith, 1971).

In this paper we report the antiviral activity *in vitro* and *in vivo* of eugenol against herpes simplex virus type 1 and 2.

MATERIALS AND METHODS

Animals. Inbred Balb/c mice, 4–6 weeks old, reared in

* Correspondence to: Dr F. Benecia, Laboratorio de Inmunoquímica, Dpto. Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Piso 4, Ciudad Universitaria, (1428) Buenos Aires, Argentina.
E-mail: fbenen@qb.fcen.uba.ar

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our own animal house, were used in all experiments. Mice were housed five per cage with sterile wood chip bedding and provided with chow pellets and tap water *ad libitum*. The animals were housed at 21°–24°C, and 40%–60% humidity with a 12 h light–dark cycle. Animals with abnormal eyes were rejected.

Cell cultures. Monkey kidney Vero cells were used. The cell line was grown in minimum essential medium (MEM) supplemented with 5% heat inactivated calf serum and 50 μg/mL gentamicin and maintained in MEM containing 2% calf serum and antibiotics.

Viruses. Herpes simplex virus type 1 (HSV-1) strain F and HSV-2 strain G were used. The virus stocks were grown in Vero cells and titrated by the plaque forming unit (PFU) method. Plaques were developed on day 2 post-infection.

Antiviral compounds. Eugenol (Sigma Chemical Co, MO) was dissolved in ethanol and stored at room temperature. It was diluted in MEM 2% to a final concentration of 1 mg/mL for bioactivity studies.

Acyclovir (ACV) (Burroughs Wellcome Co., Research Triangle Park N.C.) was dissolved in dimethylsulphoxide and diluted with MEM 1.5% to a final concentration of 1 mg/mL.

Cytotoxicity assay. Eugenol was assayed for cell toxicity prior to testing in antiviral studies. Cytotoxicity measurements were based on two parameters: (1) alteration of normal cell morphology and (2) viability of the cells present in the culture. Cell monolayers formed after 24 h of cell seeding were incubated with MEM containing different concentrations of eugenol. Treated cultures were incubated for a maximum of 72 h at 37°C. The cultures were observed daily for evidence of cytopathic effect (partial or complete loss of the monolayer, rounding or shrinkage of the cells or a granular appearance in the cytoplasm). The viability of the cells was evaluated by the Trypan blue exclusion method.

Assay of antiviral activity of eugenol. Confluent cell cultures grown in 96-well microplates were inoculated with both viruses using a multiplicity of infection of 0.1 PFU/cell. After 1 h of virus adsorption to the cells, residual virus was removed and replaced by MEM containing different concentrations of eugenol (15–250 μg/mL) in MEM (treated cultures) or MEM alone (controls). One day later virus was obtained from the cells by three cycles of freeze-thawing and then virus yield was determined by the plaque forming unit method. Briefly, Vero cells grown to confluence in 24-well microplates were infected with ten-fold dilutions of each viral suspension corresponding to the different eugenol concentrations tested. The virus was allowed to adsorb for 1 h at 37°C and then cell monolayers were washed and overlayed with MC-MEM (MEM containing 1% methylcellulose, 4000 cP). After 2 days of incubation at 37°C in 5% CO_2 in air the plates were fixed with aqueous formaldehyde, stained with 0.5% crystal violet, the virus plaques counted and virus titres were determined.

Assay of antiviral activity of eugenol-ACV combinations. Confluent cell cultures grown in 96-well microplates were inoculated with the viruses using a multiplicity of infection of 0.1 PFU/cell. After 1 h of virus adsorption to the cells, residual virus was removed and replaced by MEM containing different concentrations of eugenol and ACV in MEM (treated cultures) or MEM alone (controls). Viral cytopathogenicity was recorded as soon as it affected 100% of culture cells in the controls, i.e. at day 2, using the MTT colorimetric assay (Sudo *et al.*, 1994). Wells showing absorbance values in the MTT assay 25% lower than control cell cultures without virus were considered positive for viral replication. The antiviral activity of the compounds was expressed as 50% effective concentration (EC_{50}): the concentration required to inhibit viral cytopathogenicity by 50%. This value was calculated by extrapolation using the Reed and Muench method (1938).

A three-dimensional model to analyse eugenol–acyclovir interactions. The interactions between eugenol and acyclovir were analysed by using the three-dimensional method described by Prichard and Shipman (1990). Using the individual dose-response curves obtained for each compound, the theoretical additivity was determined using the proposed model for drugs with different target sites: $Z = X + Y(1 - X)$, where X is the antiviral activity of eugenol, Y is the antiviral activity of acyclovir and Z is the theoretical inhibition produced by the combination of both compounds. The resulting surface, which represents the calculated additivity of the drug combination, was then subtracted from the experimental surface and plotted in 3-D to reveal any regions of synergy and antagonism. These plots represent deviations from expected interactions and would reveal a horizontal plane at 0% if the interactions were purely additive. Synergistic drug interactions would appear as a peak above the plane with a height corresponding to the percent above calculated additivity. Antagonistic interactions would appear as a pit with a negative value signifying the percent below the calculated additivity.

Assay of virucidal activity. Virus samples containing 6×10^6 PFU/mL (HSV-2) and 4×10^6 PFU/mL (HSV-1) were incubated at 37°C for 1 h with MEM containing the maximum concentration of eugenol used in all experiments (250 μg/mL) (treated) or MEM alone (controls). Thereafter, samples were diluted to determine residual infectivity by the plaque forming unit method. Virucidal activity was evaluated comparing the titres of eugenol treated virus and control virus.

Inoculation of Balb/c mice. Animals were anaesthetized by intraperitoneal injection of ketamine hydrochloride 2 mg (Ketalar, Parke Davis) and 0.04 mg xylazine (Rompun, Mosby) in 0.1 mL of PBS. They were inoculated by scarification of the left cornea with a 26 gauge needle through a 5 μL drop of medium containing 10^5 PFU of HSV-1 strain F. Control mice were inoculated in the same way with a preparation of uninfected Vero cells made in the same way as the viral inoculum (mock inoculum). Eyes accidentally perforated at the time of corneal inoculation were not included in clinical or histopathological studies.

Eugenol treatment of infected mice. Mice were separated in two experimental groups receiving a 10 μL drop of different dilutions of eugenol in PBS in each eye (group 1: 1 mg/mL and group 2: 0.5 mg/mL) 24 h and 2 h

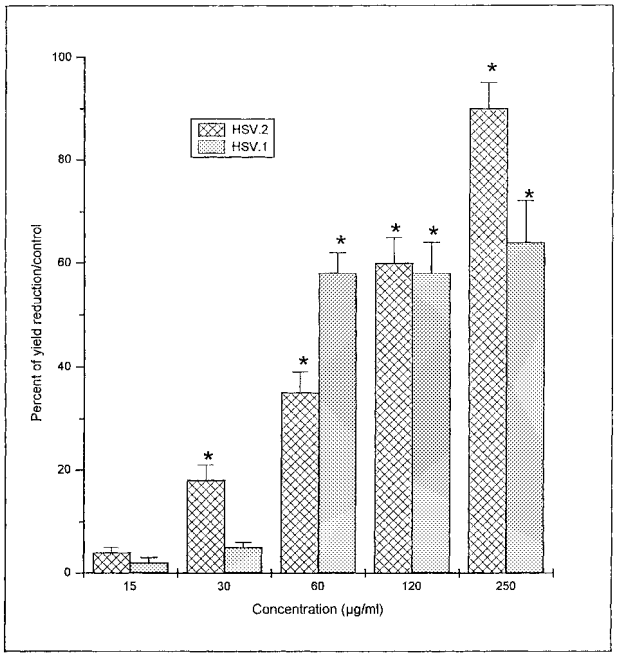

Figure 1. *In vitro* antiviral activity of eugenol. Cell cultures were inoculated with HSV-1 or HSV-2 at a multiplicity of infection of 0.1 PFU/cell. One hour later cells were washed and incubated with different concentrations of eugenol in MEM. Twenty-four hours later cells were disrupted by three cycles of freeze–thawing and virus yield was determined by the plaque forming units assay. Data are mean of three independent experiments. $*p < 0.01$.

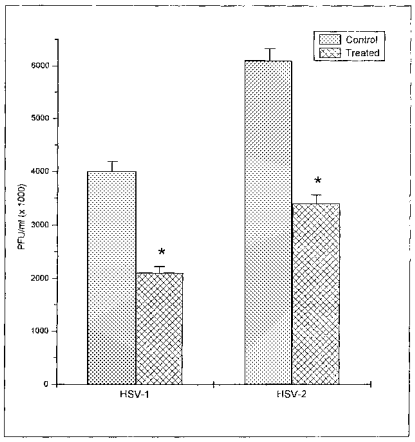

Figure 2. Virucidal activity of eugenol. Virus samples containing 6×10^6 PFU/mL (HSV-2) and 4×10^6 PFU/mL (HSV-1) were incubated at 37 °C for 1 h with MEM containing the maximum concentration of eugenol used in all experiments (250 μg/mL) (treated) or MEM alone (controls). Residual infectivity was determined by the plaque forming units assay. Data are mean of three independent experiments. $*p < 0.01$.

before virus inoculation. After infection, mice received the corresponding treatment three times a day during 5 days. In parallel, a third group received PBS instead of eugenol (control).

Clinical observations of corneal disease progression. Mice were examined using a slit lamp. The mice were clinically evaluated on days 2, 5, 7, 9, 12, 15 and 21 after corneal inoculation. Cornea, iris and lids of animals were examined for signs of disease (Irie *et al.*, 1993). Criteria for keratitis include mydriasis, iris hyperaemia, corneal ulceration, ground glass cornea and stromal opacity. Clinical evaluations were done in a masked fashion. The experiment were repeated three times to test the reproducibility of the results.

Statistical analysis. The data were analysed by using the Student's *t*-test for the *in vitro* experiments and the Chi-square method for the *in vivo* experiments.

RESULTS

Antiviral activity of eugenol

The treatment of infected cells with different concentrations of eugenol in culture medium (15–250 μg/mL) significantly diminished viral yield of both HSV-1 and HSV-2. As shown in Fig. 1, this effect was dose dependent and more pronounced on HSV-2.

On the other hand, no cytotoxic effects of eugenol were observed at the concentrations tested (15–250 μg/mL). The morphology of treated cultures was not altered by the treatment. Furthermore, according to the trypan blue exclusion method, no significant differences in the percentage of viable cells were observed between treated (250 μg/mL of eugenol) (90% ± 2.8%) and control cultures (93% ± 3.2%).

Virucidal effect of eugenol

The inhibitory effect on HSV-1 and HSV-2 replication could be partially explained by a virucidal action of eugenol since at least 50% of the infectivity was lost after mixing eugenol (250 μg/ml) with HSV-1 or HSV-2 over a period of 1 h at 37 °C (Fig. 2).

Combined antiviral effect of ACV and eugenol

We evaluated the *in vitro* antiviral activity of eugenol combined with acyclovir on HSV-1 and HSV-2 (Figs 3 and 4). The effective concentrations for 50% inhibition of HSV-1 were 25.6 μg/mL for eugenol and 0.30 μg/mL for acyclovir, while those for HSV-2 were 16.2 μg/mL and 0.27 μg/mL, respectively. The data were analysed for quantitation of synergism, additivity and antagonism of multiple drug effect by the three-dimensional model. No cytotoxicity was observed at any of the combined drug concentrations tested as determined by the MTT method. As observed in Figs 3 and 4 maximum synergism was observed at the lower acyclovir concentrations tested (0.07 and 0.15 μg/mL) and 120 μg/mL (HSV-1, Fig. 3) or 30 and 60 μg/mL (HSV-2, Fig. 4) of eugenol. As observed using eugenol alone the antiviral effect was more pronounced on HSV-2 replication where the antiviral activity was increased up to 40% above expected while for HSV-1 these values did not reach 10% above expected. On the other hand, antagonism was observed when both compounds were used at the highest concentrations tested 120 μg/mL for eugenol and 2.5 or 1.2 μg/mL for acyclovir.

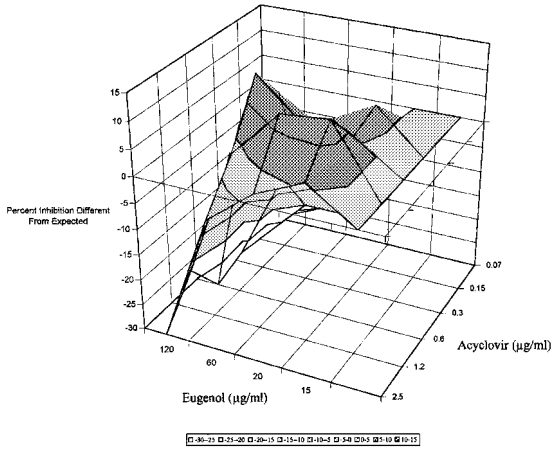

Figure 3. Antiviral activity of eugenol–ACV combinations. Cell cultures were inoculated with the HSV-1 using a multiplicity of infection of 0.1 PFU/cell. One hour later cells were washed and overlayed with MEM containing different concentrations of eugenol and ACV. Viral cytopathogenicity was recorded using the MTT colorimetric assay. The plots reveal a horizontal plane at 0% when the interactions were purely additive, synergistic drug interactions appear as a peak above the plane with a height corresponding to the percent above calculated additivity and antagonistic interactions appear as a pit with a negative value signifying the percent below the calculated additivity. Data are mean of eight independent experiments.

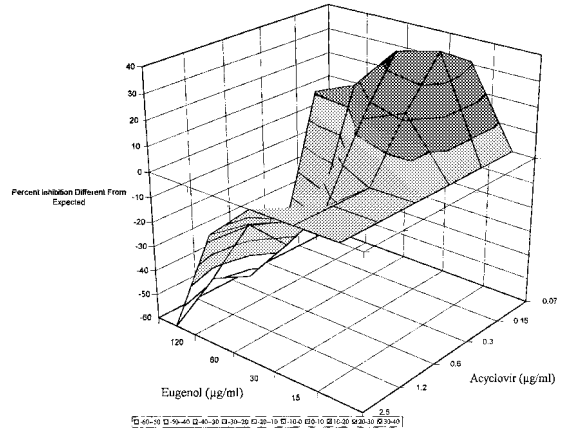

Figure 4. Antiviral activity of eugenol–ACV combinations. Cell cultures were inoculated with HSV-2 using a multiplicity of infection of 0.1 PFU/cell. One hour later cells were washed and overlayed with MEM containing different concentrations of eugenol and ACV. Viral cytopathogenicity was recorded using the MTT colorimetric assay. The plots reveal a horizontal plane at 0% when the interactions were purely additive, synergistic drug interactions appear as a peak above the plane with a height corresponding to the percent above calculated additivity and antagonistic interactions appear as a pit with a negative value signifying the percent below the calculated additivity. Data are mean of eight independent experiments.

Effect of eugenol treatment on herpetic keratitis

The treatment of corneal infected animals with eugenol significantly altered the development of stromal keratitis only at the highest concentration tested, 1 mg/mL, while 0.5 mg/mL had no effect on it. As shown in Fig. 5, the treatment provoked a delay in the outcome of disease signs but, by day 10 post infection (3 days later than control group), all the mice were ill. These results were confirmed by microscopic examination of haematoxylin and eosin stained histopathological eye sections obtained by standard procedures (Babu *et al.*, 1996) (data not shown).

DISCUSSION

The ethnopharmacognostic approach to the search for bioactive compounds can lead to the identification of novel substances as well as to known molecules not previously reported as having biological activity. Eugenol is the principal component of clove oil and is also present in other essential oils. It has been used for years in dentistry. In the present study we demonstrate eugenol possesses antiviral activities *in vitro* and *in vivo* against human herpesvirus. *In vitro* the effect was dose dependent and more marked on HSV-1. It is noteworthy to comment that this effect was at least partially due to a virucidal activity of eugenol in agreement with previous data indicating that clove oil exerts a virucidal effect on HSV-1, disabling the viral lipidic envelope. On the other hand, some studies have shown that eugenol induced glutathione S-transferase (GST) in rat liver *in vivo* (Rompelberg *et al.*, 1993) and Palamara *et al.* (1995) have reported an antiviral activity of glutathione, inhibiting the *in vitro* replication of HSV-1. Thus, its

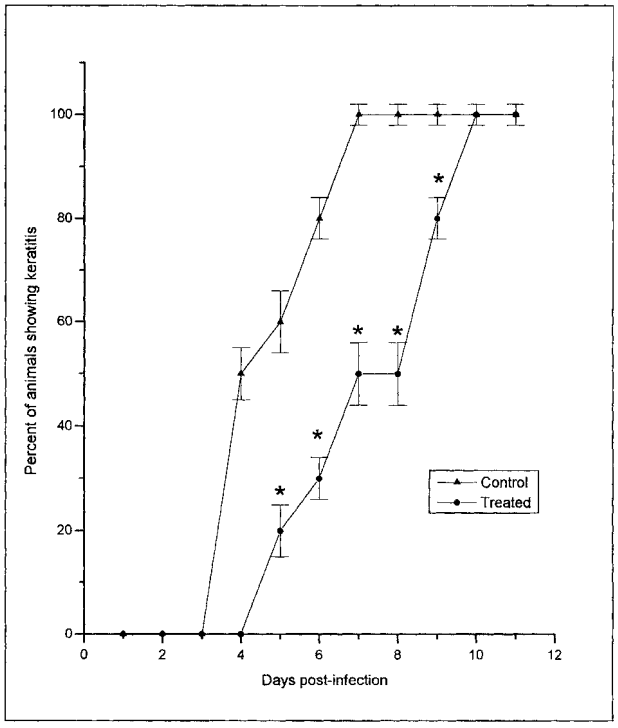

Figure 5. *In vivo* antiviral activity of eugenol. Mice were separated in two experimental groups (10 animals each) receiving a 10 µl drop of eugenol (1 mg/mL) in PBS or the diluent (control) 24 h and 2 h before virus inoculation. After infection, mice received the corresponding treatment three times a day for 5 days. At different days post-infection cornea, iris and lids of animals were examined for signs of disease. Criteria for keratitis include mydriasis, iris hyperaemia, corneal ulceration, ground glass cornea and stromal opacity. Clinical evaluations were done in a masked fashion. Data are mean of three independent experiments. $*p < 0.01$ according to the Chi square method.

tempting to speculate that the overall antiviral activity of eugenol could be also due to an indirect effect of its reported modulatory influences on cellular GST activities and acid-soluble sulphydryl levels.

In recent years several attempts have been made to discover synergistic combinations of antiviral compounds. The advantages of such combinations are reduced doses of potentially toxic compounds, reduced probability for the emergence of drug resistant viruses and increased antiviral potency (Barquero *et al.*, 1997). In this work we found that ACV and eugenol synergistically inhibited HSV replication *in vitro*, which supports the fact that both compounds exert their antiviral effect on different viral targets: ACV on DNA replication and eugenol as discussed above. The synergism was confirmed using a three-dimensional model which is considered to be one of the more powerful methods for analysing these interactions (Prichard and Shipman, 1990).

Finally, eugenol proved to be effective in an *in vivo* model of HSV-1 infection. Eugenol treatment significantly delayed the development of herpetic keratitis in the cornea of HSV-1 infected mice, thus indicating that this compound could be useful in the treatment of ocular herpetic infections. Eugenol may not be useful for internal applications since the antiviral effect is at least in part due to virucidal activity, but taking into account the results of *in vivo* experiments plus its stability at room temperature it could be used for topical treatments, particularly in combination with other drugs, since its antiviral activity is slight compared with other drugs such as acyclovir.

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