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Effect of Eucalyptus Essential Oil on Respiratory Bacteria and Viruses

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Abstract The activity of *Eucalyptus globulus* essential oil was determined for 120 isolates of *Streptococcus pyogenes*, 20 isolates of *S. pneumoniae*, 40 isolates of *S. agalactiae*, 20 isolates of *Staphylococcus aureus*, 40 isolates of *Haemophilus influenzae*, 30 isolates of *H. parainfluenzae*, 10 isolates of *Klebsiella pneumoniae*, 10 isolates of *Stenotrophomonas maltophilia* and two viruses, a strain of adenovirus and a strain of mumps virus, all obtained from clinical specimens of patients with respiratory tract infections. The cytotoxicity was evaluated on VERO cells by the MTT test. The antibacterial activity was evaluated by the Kirby Bauer paper method, minimum inhibitory concentration, and minimum bactericidal concentration. *H. influenzae*, *parainfluenzae*, and *S. maltophilia* were the most susceptible, followed by *S. pneumoniae*. The antiviral activity, assessed by means of virus yield experiments titered by the end-point dilution method for adenovirus, and by plaque reduction assay for mumps virus, disclosed only a mild activity on mumps virus.

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Introduction

In traditional popular medicine, *Eucalyptus* spp. essential oil has been traditionally used to treat respiratory tract disorders and infections; inhalation of *Eucalyptus* derivatives has been used to treat pharyngitis, bronchitis, and sinusitis. Over the past few years, the interest in natural medicine has been increasing in industrialized societies also. Consequently, the scientific attention to this field is expanding. Some research has been conducted on the antibacterial and antiviral activity of myrtaceous *Eucalyptus* oil showing some efficacy [2, 6, 7–10]. *Streptococcus pyogenes, pneumoniae, agalactiae, Staphylococcus aureus, Haemophilus influenzae, Parainfluenzae*, and *Stenotrophomonas maltophilia* are the most important causes of respiratory tract infections, and the emergence of resistance to antibiotics is a serious public health problem. The present study investigated the antibacterial and antiviral activity of *Eucalyptus globulus* essential oil against pathogens isolated from patients with respiratory tract infection.

Materials and Methods

Eucalyptus Oil

A commercial preparation of *Eucalyptus globulus* was used in all of the experiments (Orlandi Erboristerie s.r.l., Reggio Emilia, Italy). No indication of the oil concentration was reported on the package or the composition.

Bacterial Strains

Streptococcus pyogenes (120 strains), Streptococcus pneumoniae (20 strains), Streptococcus agalactiae (40

strains), Staphylococcus aureus (20 strains), Haemophilus influenzae (40 strains), Haemophilus parainfluenzae (30 strains), Klebsiella pneumoniae (10 strains), and Stenotrophomonas maltophilia (10 strains) isolated from throat swabs and other specimens were submitted to the Laboratory of Clinical Microbiology, Arcispedale S. Maria Nuova, Reggio Emilia for microbiological analysis.

Cells and Viruses

The green monkey kidney cell line VERO was used to grow both adenovirus (ADV) and mumps virus (MV). Cells were cultured in Minimum Essential Medium (MEM) added with 10% (growth medium) or 5% (maintenance medium) fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and incubated at 37°C with 5% CO_2.

ADV and MV were clinical strains adapted to grow in cells with several in vitro passages. A same stock of each virus kept frozen at −80°C was used in all the experiments.

Kirby Bauer Paper Method

The experiment was performed with a bacterial inoculum of 0.5 McFarland; Mueller-Hinton (MH) with 5% sheep blood was inoculated with each bacterial strain. Each oil (10 μl) was applied to a sterile filter paper disc (6-mm diameter) placed on the surface of inoculated plates; duplicate plates for each oil were used. After overnight incubation at 37°C, the inhibition zones were measured. Control plates was prepared by placing sterile water for negative controls.

Determination of the Minimum Inhibitory Concentration

The microbroth dilution method was performed to determine the minimum inhibitory concentration (MIC). A final concentration of 0.5% Tween-20 was incorporated into MH broth to enhance oil solubility. Briefly, a series of twofold dilutions of eucalyptus oil was prepared in broth with 0.5 Tween-20 and seeded in a 96-well culture plate, and was then inoculated with bacterial inoculum. MH broth, with 0.5% Tween-20 but no oil, was used as positive growth control. Inoculated microplates were incubated at 37°C for 24 h. The MIC was determined as the lowest concentration of oil inhibiting the growth of each organism.

Determination of the Minimum Bacteriocidal Concentration

The minimum bacteriocidal concentration (MBC) was determined by transferring diluted broth from each well of microdilution plate to a Petri dish containing MH agar with 5% sheep blood. The plates were incubated at 37°C for 24 h. The MBC was read as the lowest concentration of oil that prevented growth of more than one colony on subculture.

Cytotoxicity Assays

The VERO cell line was used to test the cytotoxicity of the essential oils under study. Cells were cultured in E-MEM with 10% FCS as growth medium and with 5% FCS as maintenance medium. In order to dissolve *Eucalyptus globulus* essential oil in the cell medium, it was added with 10% Tween-20 and the suspension obtained was vigorously vortexed before dilution. Serial dilutions of this emulsion from 1 μl/ml to 0.05 μl/ml were prepared in maintenance medium. Twenty-four-hour growth VERO cells in a 96-well plate were challenged with 250 μl of each dilution and daily observed with a light microscope to check the cell condition. Each dilution was tested in triplicate and compared to control untreated cells. The effects on cell viability of the oil concentrations disclosing no apparent cytotoxic effects on the cell cultures at light microscope observation were tested by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test [1]. Cells were treated with *Eucalyptus globulus* essential oil for 48 h; then the MTT stain was added to each well at a 0.5 mg/ml final concentration. After 3-h incubation, the insoluble formazan developed in viable cells was solubilized by adding 0.1N HCl in absolute isopropanol. Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm. Each dilution was always tested in triplicate, and in each set of experiments three control wells without drug were included.

Antiviral Assay

Antiviral activity was ascertained by means of viral yield assays for ADV and with plaque reduction assay for MV. Twenty-four-hour growth VERO cells were infected at a multiplicity of infection (MOI) of 0.01 50% tissue culture infected dose ($TCID_{50}$)/cell. After 1-h adsorption at 37°C, the inoculum was removed, the plates were washed with phosphate-buffered saline (PBS), and the essential oil added was to the maintenance medium. After 2-day

Table 1 Antibacterial activity of *Eucalyptus globulus* essential oil

	S. pyogenes	*S. agalactiae*	*S. pneumoniae*	*S. aureus*	*H. influenzae*	*H. parainfluenzae*	*K. pneumoniae*	*S. maltophila*
Kirby Bauer[a]	5	3	15	2	28	27	0	20
MIC[b]	50	50	25	50	1.25	1.25	/	1.25
MBC[b]	50	50	25	50	1.25	1.25	/	1.25

[a] Mean values expressed as mm

[b] Values expressed as µl/ml

MIC minimum inhibitory concentration, *MBC* minimum bactericidal concentration

incubation, the plates were frozen and thawed three times and the viral yield was titered by end-point titration assay. In this case, 10-fold dilutions of each cell lysate were seeded onto 24-h-growth VERO cells in a 96-well culture plate; after 2 days the titer, expressed as $TCID_{50}$/ml, was read, taking account of the final dilution showing the typical viral cytopathic effect, and the results were elaborated using the Reed and Muench formula [5]. Plaque assays were carried out as follows. Twenty-four-hour growth VERO in 24-well tissue culture plates were infected with MV at a MOI 50 plaque-forming units/well. After one adsorption, virus inoculum was removed, the plates were washed with PBS, and the maintenance medium with or without (control cultures) the essential oil under study and with 0.6% human γ-globulin was added. After 72-h incubation, the plates were fixed with methanol, stained with Giemsa, and the plaques were counted.

Results and Discussion

Table 1 shows the results of the antibacterial assays in terms of Kirby Bauer, MIC, and MBC. Inhibition ranged from 2 to 28 mm.

H. influenzae, H. parainfluenzae, and *S. maltophilia* were the most susceptible, followed by *S. pneumoniae* and *S. agalactiae*. *K. pneumoniae* did not show susceptibility to eucalyptus oil.

MIC values did not exhibit substantial variations when compared to the Kirby Bauer paper method. MIC, ranging between 50 µl/ml and 1.25 µl/ml, indicated that there was identity between MIC and MBC. The highest activity was obtained, with MIC values of 1.25 µl/ml, for *H. influenzae*, *H. parainfluenzae*, and *S. maltophilia*.

The cytotoxicity tests showed that the highest nontoxic concentration on VERO cells was 0.25 µl/ml. This concentration was used in the antiviral assays. As shown in Figure 1, eucalyptus oil showed a mild antiviral activity against only MV, but not against ADV. Literature data provide evidence of antiherpesvirus activity of eucalyptus oil [8]. Because both herpes simplex virus and MV are enveloped viruses, it can be speculated that this antiviral activity, at least partially, may be due to a direct action on the virus particles during the extracellular phase of virus cycle.

In the literature, antibacterial activity of eucalyptus oil against bacteria causing respiratory tract disorders has been reported [3, 4, 6, 7]. In our hands, the antibacterial activity was gained at concentrations higher than those not cytotoxic: therefore, it is difficult to discriminate a specific antimicrobic action from toxic activity. Nevertheless, comparison of the results of this study with previously published data is difficult because the composition of plant products is known to vary according to local climatic conditions and soil composition and to extraction techniques. Moreover, the results obtained may differ because of the method used to assess antimicrobial activity. Herbal commercial products rarely report the oil concentration and never the chemical composition of the plant extract.

This suggests that the lack of standardization of these types of products, i.e., different methods of preparation of essential oils, together with variability in plant chemical profiles, has an impact on whether or not the essential oil is

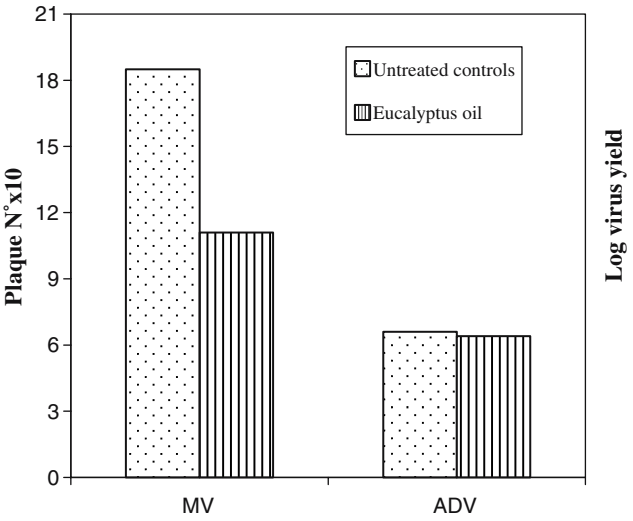

Fig. 1 The antiviral activity of eucalyptus essential oil was assessed by plaque reduction assay for mumps virus (MV) and by virus yield assay, titered by end-point titration, for adenovirus (ADV). Eucalyptus oil was used at a concentration of 0.25 µl/ml

of use as an antimicrobial agent in clinical practice. This indicates the need for stricter laws for herbal products that are sold without many controls and do not require any analytical report. The data obtained in this investigation suggest that eucalyptus oil deserve further studies, especially on a wider spectrum of viruses.

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