In Vitro Inhibition of Cytopathic Effect of Influenza Virus and Human Immunodeficiency Virus by Bamboo Leaf Extract Solution and Sodium Copper Chlorophyllin

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ABSTRACT

Background Although the link between oral and oropharyngeal health status and susceptibility to infection has long been recognized, there is a limit to the selection of antiseptics for oral care.

Methods Madin-Darby canine kidney (MDCK) cells were exposed to influenza virus and cultured in the presence or absence of test reagents: bamboo leaf extract solution and sodium copper chlorophyllin. MDCK cells were pre-incubated with the reagents to assess the inhibitory activity at adsorption (viral attachment). Similarly, anti-HIV activity and the inhibitory mechanism at adsorption were assessed by MT-2 cell culture system. Mixture of HIV and bamboo leaf extract solution was fixed and examined by transmission electron microscopy.

Results The 50% inhibitory concentration (IC₅₀) of bamboo leaf extract solution against influenza virus and the 50% cytotoxic concentration (CC₅₀) in MDCK cells of the solution lay between 0.0313–0.0625% and 0.5–1.0%, respectively. The solution inhibited the influenza virus adsorption at the concentration of 0.5% (P < 0.05). The values of IC₅₀ and CC₅₀ of sodium copper chlorophyllin lay between 50–100 µM and 200–400 µM, respectively. This inhibited the virus adsorption at 200 µM (P < 0.05). The bamboo leaf extract solution showed values of IC₅₀ against HIV and CC₅₀ in MT-2 cells at around 0.0313% and between 0.25–0.5%, respectively. This solution inhibited HIV adsorption at 1.25% (P < 0.05). The IC₅₀ and CC₅₀ of sodium copper chlorophyllin lay between 50–100 µM and 200–400 µM, respectively. Sodium copper chlorophyllin inhibited HIV adsorption at 2.5 mM (P < 0.05). HIV particles survived after the exposure to 0.5% bamboo leaf extract solution.

Conclusion Sodium copper chlorophyllin exerted antiviral activities against influenza virus and HIV as the major ingredient of bamboo leaf extract solution by blocking adsorption. This mechanism of action is different completely from the one of povidone-iodine.

Key words Adsorption; bamboo leaf extract solution; HIV; influenza; human; sodium copper chlorophyllin

The accumulation of dental plaque and colonization of oral surfaces with respiratory pathogens serves as a reservoir for recurrent lower respiratory tract infections. Indeed, professional oral health care by dental hygienists is effective in preventing respiratory infections in elderly persons requiring nursing care. Although the linkage between oral and oropharyngeal health status and susceptibility to infection has long been recognized, there is a limit to the selection of antiseptics for oral care.

Povidone-iodine is one such antiseptic and considered to have the broadest spectrum of antimicrobial action compared with other common antiseptics. Oxidative potency of povidone-iodine enables destruction of various structures and enzymes of microbes and viruses. However, the antiseptic has a limitation leading to the possible cytotoxic potential to mammalian cells through this mechanism of pathogen killing. Therefore, further exploration is required for the development of new materials that have different anti-pathogenic mechanisms.

Influenza viruses cause a viral respiratory infection particularly among high-risk individuals, such as children, aged persons, and patients with chronic disorders. Some reports indicate that the unclean condition in the oral cavity promotes influenza infection, and good oral hygiene minimizes the severity of respiratory illness. Human immunodeficiency virus (HIV) infected approximately 34 million individuals causing acquired immune deficiency syndrome (AIDS) worldwide. One investigation found that 76.7% of HIV-infected individuals showed oral symptoms among tested AIDS cases. Among these HIV-infected individuals, gum bleeding is observed frequently due to corruption of mucous membrane of the oral cavity after the progress of caries. Such bleeding from the oral cavity may increase the possibility of HIV transmission to partners. Therefore, it is indispens-
able for such individuals to efficiently improve their own oral care with potent antimicrobial activity for prevention of infection and disease development.

In the present study, antiviral activities of a plant extract and its ingredient against influenza virus and HIV were assessed for antiseptic use.

**MATERIALS AND METHODS**

**Influenza virus cytopathic assay**

Madin-Darby canine kidney (MDCK) cells were seeded (30,000 per well) in 96-plate and incubated for approximately 24 hours. Then, the semi-confluent cells were washed twice with phosphate-buffered saline, exposed to influenza virus [A/Tottori/ST215/2009(H1N1)] at a dose of 10^8 copies per well and cultured in the presence or absence of various concentrations of test reagents: bamboo leaf extract solution (kindly provided by Environmental Plant Industry, Yonago, Japan), sodium copper chlorophyllin (Wako, Osaka, Japan), and povidone-iodine (‘Isodine gargle’, Meiji, Tokyo, Japan). The culture was maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10 µg/mL trypsin (Beckton Dickinson, Tokyo, Japan), 0.2% heat-inactivated bovine serum albumin, 200 units/mL penicillin G, and 100 µg/mL streptomycin at 37 °C under a 5% CO2/95% air atmosphere for four days. Control cells were not exposed to the virus. The total viable cells were counted by trypan blue dye exclusion method on day 4.

MT-2 cells were pre-incubated to the bamboo leaf extract solution (up to 10%), sodium copper chlorophyllin (up to 5 mM) or test reagent-free culture medium for 10 min to assess the inhibitory activity at adsorption. The pre-incubated cells were washed extensively to be able to eliminate antiviral activity of test reagents remaining in the culture medium, and then they were exposed to HIV-1LAI (1.3x10^4 TCID50 per cell). In addition to these pre-incubated cells, control wells were prepared to evaluate growth of the cells in a virus-free condition. Then, virus-exposed and mock-exposed cells were cultured in the absence of test reagents for three days. The total viable cells were counted on day 3.

**Transmission electron microscopy**

The HIV-1LAI (50 µL, 4,000 TCID50 per mL) was subjected to Viro-adembeads (ADEMTEC, Pessac, France) according to manufacturer’s instruction. The HIV-1LAI captured with Viro-adembeads was incubated with an equal volume of bamboo leaf extract solution at an ambient temperature for 5–10 min. The mixture was then fixed with 2.5% glutaraldehyde at 4 °C for more than one hour, washed twice with 70% ethanol and re-suspended with phosphate buffered saline (100 µL). The fixed virus particles were postfixed in 1% osmium tetroxide, and stained with 1% uranyl acetate for 1h. They were then dehydrated in graded ethanol, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan).

**Statistical analysis**

Statistical analyses were performed using the JMP 9 software (SAS Institute, Cary, NC). The increase in the total viable cells was evaluated by Dunnett’s test, and P < 0.05 was regarded as statistically significant.

**RESULTS**

**Anti-influenza virus activity of bamboo leaf extract solution and sodium copper chlorophyllin and its inhibitory mechanism**

Original bamboo leaf extract solution containing 3.3–3.9 mM sodium copper chlorophyllin was defined as 100% concentration in the present experiment. The 50% inhibitory concentration (IC50) of bamboo leaf extract solution lay between 0.0313 and 0.0625%. The 50% cytotoxic concentration (CC50) of the solution lay between 0.5
and 1% (Fig. 1A). The solution exhibited pretreatment effect and inhibited the influenza virus adsorption at a concentration of 0.5% \((P < 0.05)\) (Fig. 1B). The values of IC\(_{50}\) and CC\(_{50}\) of sodium copper chlorophyllin lay between 50–100 µM and 200–400 µM, respectively (Fig. 1C). Sodium copper chlorophyllin also exhibited virus adsorption at 200 µM \((P < 0.05)\) (Fig. 1D).

**Anti-HIV activity of bamboo leaf extract solution and sodium copper chlorophyllin and its inhibitory mechanism**

The bamboo leaf extract solution showed values of IC\(_{50}\) and CC\(_{50}\) at around 0.0313% and between 0.25–0.5%, respectively (Fig. 2A). This solution exhibited pretreatment effect on the inhibition of HIV adsorption at the concentration of 1.25% \((P < 0.05)\) (Fig. 2B). As with the anti-influenza virus activity, the IC\(_{50}\) and CC\(_{50}\) of sodium copper chlorophyllin lay between 50–100 µM and 200–400 µM, respectively (Fig. 2C). Sodium copper chlorophyllin also exhibited pretreatment effect and inhibited HIV adsorption at 2.5 mM \((P < 0.05)\) (Fig. 2D).

**Anti-influenza virus and anti-HIV activities of povidone-iodine ‘Isodine gargle’**

No antiviral activity can be observed against influenza virus and HIV although the povidone-iodine concentration increased to a level close to cytotoxic concentration: 0.125–0.25% of available iodine in MDCK cell culture (Fig. 3A) and 0.0313–0.0625% in MT-2 cells (Fig. 3B).

**Morphology of HIV particles after exposure to bamboo leaf extract solution**

Stability of HIV particles was analyzed in the concentration with potent antiviral activity in the HIV culture. Intact HIV particles could be observed even in the presence of 0.5% bamboo leaf extract solution (Fig. 4). This concentration is sufficient to exhibit an anti-HIV activity in culture (0.5%, in Fig. 2A), and corresponds to 16-fold of IC\(_{50}\) and around CC\(_{50}\).

**DISCUSSION**

Antiviral activity of bamboo leaf extract solution was recognized as an agent that fights against the influenza virus.
Fig. 2. Inhibition of the cytopathic effect of HIV by bamboo leaf extract solution and sodium copper chlorophyllin. The entire procedure was carried out similarly to the inhibition assay of cytopathic effect of influenza virus except susceptible cells and viral inoculum: MT-2 cells and HIV. *P < 0.05 by Dunnett's test. MDCK, Madin-Darby canine kidney; HIV, Human immunodeficiency virus.

Fig. 3. Inhibition of the cytopathic effect of influenza virus and HIV. A: MDCK cells were exposed to influenza virus and cultured in the presence or absence of povidone-iodine (solid column). Control cells were not exposed to the virus (open column). The reagent concentration was increased up to the cytotoxic level (0.25%). B: Similarly, MT-2 cells were exposed to HIV and cultured. There was no viable cell at 0.125% on day 3. MDCK, Madin-Darby canine kidney; HIV, Human immunodeficiency virus.
Bamboo leaf/Chlorophyllin as antivirals

The inhibitory mechanism has been estimated as non-specific interaction against several different microbes. The mechanism of action must be different from the one of povidone-iodine. The extract solution and sodium copper chlorophyllin kept antiviral activities for the entire culture period, but povidone-iodine did not inhibit cytopathic effect even at a subtoxic concentration. Povidone-iodine must exert antiviral activity for short instances such as one minute incubation, and give irreversible damage to viral particles as summarized before. This difference was also supported by the fact that HIV particles were not destroyed after 5–10 min exposure to subtoxic concentration of bamboo leaf extract solution, and could be recognized by electron microscopy. However, additional morphological examination has to be carried out also in the presence of povidone iodine for further discussion of the difference between antiviral mechanisms of the bamboo leaf extract and povidone iodine.

In the current study, sodium copper chlorophyllin exerted antiviral activities against influenza virus and HIV as the major ingredient of bamboo leaf extract solution by blocking viral-cell interaction (adsorption step) and interfering with the internalization of viruses and resultant replication. This mechanism of action is completely different from the one occurring with povidone-iodine.

Acknowledgments: The authors wish to thank the Environmental Plant Industry for providing bamboo leaf extract. The authors declare no conflict of interest.

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