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 chrolophyllin. 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 (IC_{50}) of bamboo leaf extract solution against influenza virus and the 50% cytotoxic concentration (CC_{50}) in MDCK cells of the solution lay between 0.0313–0.0625% and 0.5–1.0%. The solution inhibited the influenza virus adsorption at the concentration of 0.5% ($P < 0.05$). The values of IC_{50} and CC_{50} 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 IC_{50} against HIV and CC_{50} 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 IC_{50} and CC_{50} 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.[1–5] Indeed, professional oral health care by dental hygienists is effective in preventing respiratory infections in elderly persons requiring nursing care.[6–9] 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.[10, 11]

Povidone-iodine is one such antiseptic and considered to have the broadest spectrum of antimicrobial action compared with other common antiseptics.[12, 13] Oxidative potency of povidone-iodine enables destruction of various structures and enzymes of microbes and viruses.[14] However, the antiseptic has a limitation leading to the possible cytotoxic potential to mammalian cells through this mechanism of pathogen killing.[15–17] 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,[18] and good oral hygiene minimizes the severity of respiratory illness.[3, 19] 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.[20] 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.[21–23] Such bleeding from the oral cavity may increase the possibility of HIV transmission to partners. Therefore, it is indispens-

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Abbreviations: CC_{50}, 50% cytotoxic concentration; D-MEM, Dulbecco's modified eagle medium; HIV, Human immunodeficiency virus; IC_{50}, 50% inhibitory concentration; MDCK, Madin-Darby canine kidney; $TCID_{50}$, Median tissue culture infectious dose

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 (D-MEM) 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 34 °C under a 5% CO_2/95% air atmosphere for three days. Control cells were not exposed to the virus. The total viable cells were counted by a sensitive colorimetric assay (Cell Counting Kit-8, Wako, Osaka) on day 3.

The semi-confluent MDCK cells were pre-incubated with the bamboo leaf extract solution (0.5 and 1%), sodium copper-chlorophyllin (200 and 400 μM) or test reagent-free culture medium for 10 min to assess the inhibitory activity at adsorption (viral attachment). The pre-incubated cells were washed extensively to be able to ignore antiviral activity of the remaining test reagents in the culture medium, and then exposed to the influenza virus ($5x10^7$ copies per well). 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.

HIV cytopathic assay

MT-2 cells (10^5 cell per mL) were exposed to of HIV-1_{LAI} at $1.3x10^{-4}$ median tissue culture infectious dose ($TCID_{50}$) per cell, and cultured in the presence or absence of various concentrations of bamboo leaf extract solution, sodium copper chlorophyllin, and povidone-iodine on day 0. The culture was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 200 units/mL penicillin G, and 100 μg/mL streptomycin at 37 °C under a 5% CO_2/95% 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-1_{LAI} ($1.3x10^{-4}$ $TCID_{50}$ 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-1_{LAI} (50 μL, 4,000 $TCID_{50}$ per mL) was subjected to Viro-adembeads (ADEMTEC, Pessac, France) according to manufacturer's instruction. The HIV-1_{LAI} 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 resuspended 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 Dunett'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 (IC_{50}) of bamboo leaf extract solution lay between 0.0313 and 0.0625%. The 50% cytotoxic concentration (CC_{50}) 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

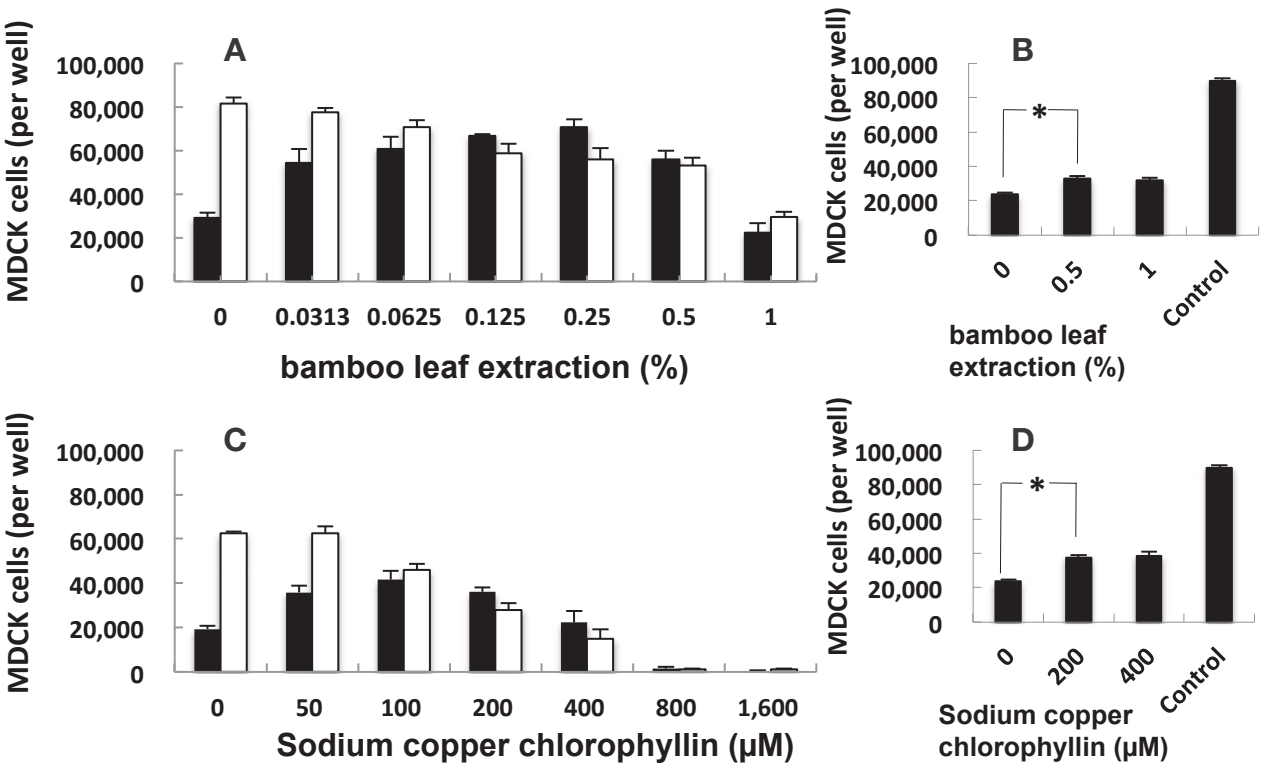

Fig. 1. Inhibition of the cytopathic effect of influenza virus by bamboo leaf extract solution and sodium copper chlorophyllin. **A**: MDCK cells were exposed to influenza virus, and cultured in the presence or absence of various concentrations of bamboo leaf extract solution (solid column). Control cells were not exposed to the virus (open column). Data were expressed as mean ± standard error ($n = 4$). **B**: MDCK cells were pre-incubated to the bamboo leaf extract solution or medium and then exposed to the influenza virus after extensive washing. Cell culture was carried out in the absence of test reagent. The total viable cells were counted on day 3. **C**: The viable MDCK cells exposed to influenza virus were cultured in the presence or absence of the sodium copper chlorophyllin. **D**: Pre-incubated MDCK cells to the sodium copper chlorophyllin or medium were exposed to the influenza virus after extensive washing. Cell culture was carried out in the absence of test reagent. *$P < 0.05$ by Dunnett's test. MDCK, Madin-Darby canine kidney.

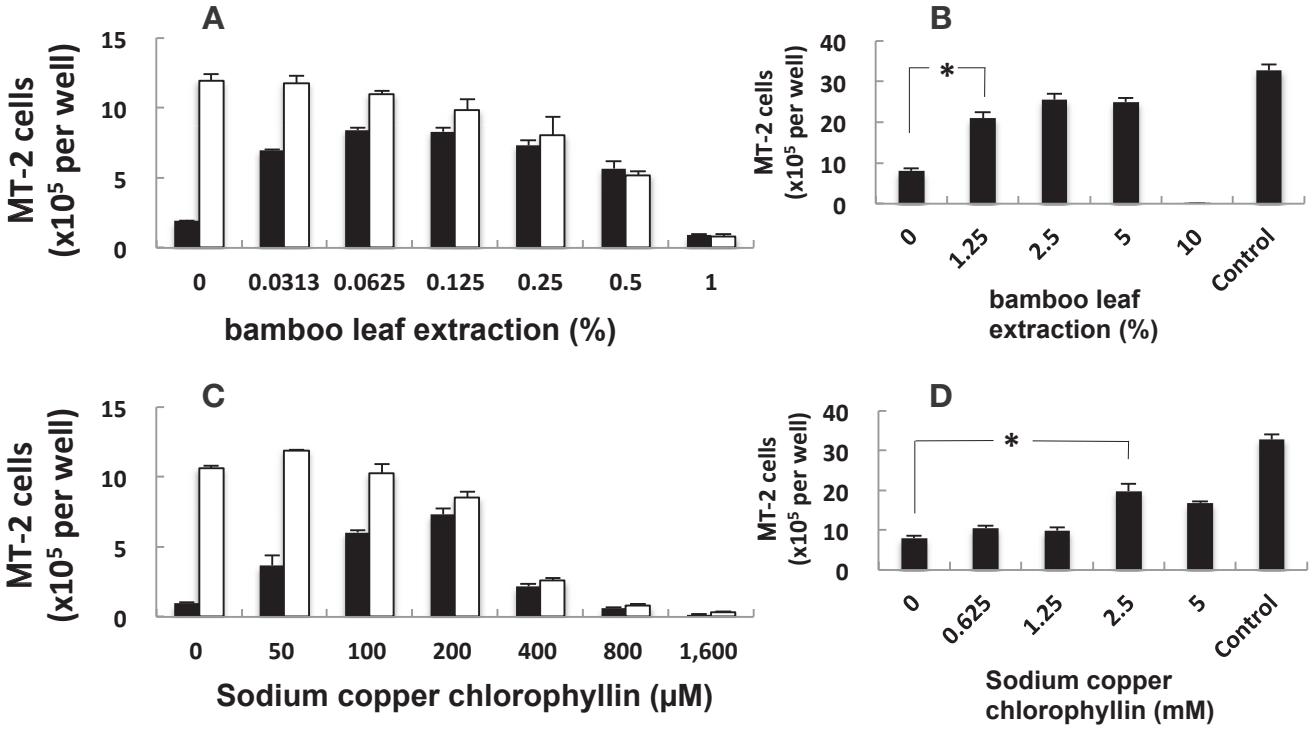

Fig. 2. Inhibition of the cytopathic effect of HIV by bamboo leaf extract solution and sodium copper chlorophyllin. The entire procedure was carried out similarly to the inhibition assay of cytopathic effect of influenza virus except susceptible cells and viral inoculum: MT-2 cells and HIV. *$P < 0.05$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

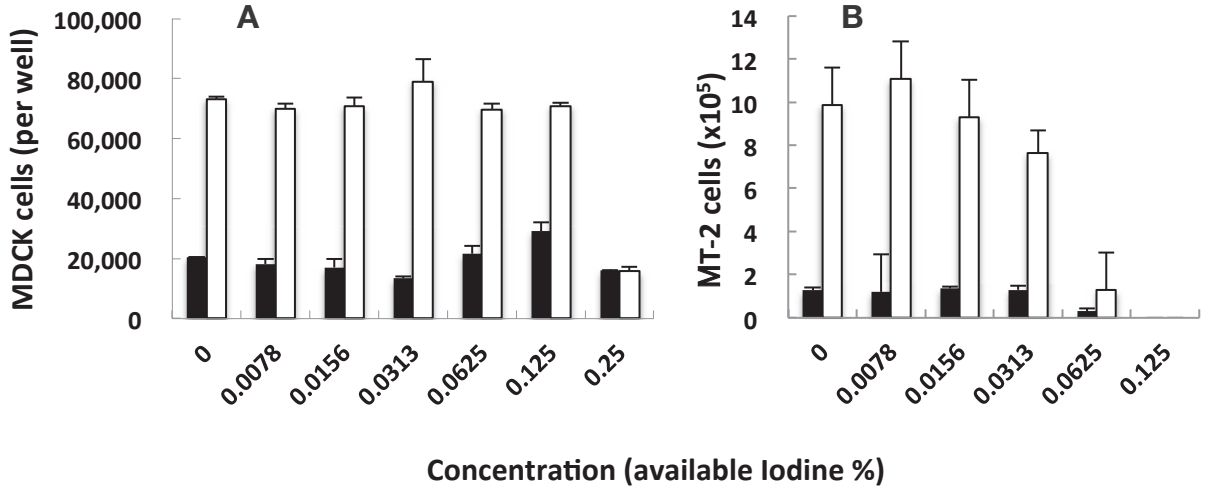

Fig. 3. Inhibition of the cytopathic effect of influenza virus and HIV. **A**: MDCK cells were exposed to influenza virus and cultured in the presence or absence of povidone-iodine (solid column). Control cells were not exposed to the virus (open column). The reagent concentration was increased up to 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.

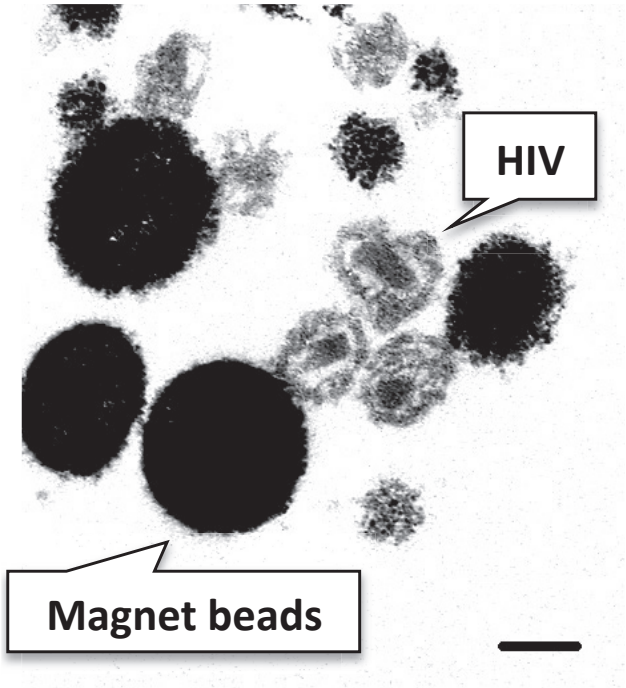

Fig. 4. Intact HIV particles after exposure to higher concentration of bamboo leaf extract. Typical HIV particles approximately 120 nm in diameter with a conical capsid can be seen even after incubation with 0.5% bamboo leaf extract solution for more than five minutes. The diameter of magnet beads ranges from 100–500 nm according to manufacturer (Ademteck). Bar = 100 nm. MDCK, Madin-Darby canine kidney; HIV, Human immunodeficiency virus.

virus and HIV, and was exhibited presumably by inhibition of viral adsorption to cells as reported for other natural ingredients, such as black tea, coffee, Morinda citrifolia leaves, manuka honey and bananas.[24–28] One ingredient, sodium copper chrolophyllin, also had antiviral activity against influenza and HIV through the same antiviral mechanism of the extract solution. The activity of this extract solution may be due to this ingredient. The IC_{50} of the extract solution lay between 0.0313 and 0.0625% and must contain 116–230 μM sodium copper chlorophyllin. Similarly, the IC_{50} of the compound, sodium copper chrolophyllin, lay between 50–100 μM. These two IC_{50} values of sodium copper chlorophyllin as ingredients and compound were almost in the same concentration range. These results suggested sodium copper chlorophyllin exerted antiviral activities against influenza and HIV as the major ingredient of bamboo leaf extract solution.

As described above, the extract solution and sodium copper chlorophyllin exerted antiviral activities by inhibiting the virus-to-cell interaction on the cell surface, as this inhibitory mechanism has been estimated as non-specific interaction against several different microbes.[29–32] The mechanism of action must be different from the one of povidone–iodine.[12, 33] The extract solution and sodium copper chrolophyllin 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.[12] 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.

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The authors declare no conflict of interest.

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