BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **225,** 22–26 (1996)
ARTICLE NO. 1125

Antiviral Effect of *Sanicula europaea* L. Leaves Extract on Influenza Virus-Infected Cells

Kadir Turan,* Kyosuke Nagata,† and Avni Kuru*,[1]

**Department of Biology, Faculty of Science, Istanbul University, 34459, Vezneciler, Istanbul, Turkey; and †Department of Biomolecular Engineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology 4259 Nagatsuta, Midori-ku, Yokohama 226, Japan*

Received May 29, 1996

We have investigated the influence of *Sanicula europaea* L. extracts on influenza virus growth in MDCK cells. Fractions I, II, and III separated from *Sanicula* extract with Sephadex column chromatography were found to be non-toxic against MDCK cells. The growth of influenza A/PR/8/34 was completely inhibited by these fractions, while that of influenza B/Lee/40 was not affected. Fractions II and III have been shown not to have a direct virucidal activity on influenza A/PR/8/34. Influenza A/Vic/1/75 produced microscopic plaques in the presence of the extract. *In vitro* RNA synthesis with viral RNA-dependent RNA polymerase was also inhibited by a water soluble extract of *Sanicula.* These observations suggest that the *Sanicula* extract contains an anti-influenza virus substance.

Influenza viruses characteristically cause recurrent epidemics of disease in the human population. This is largely due to the continual changes which occur in the antigenic properties of virus surface glycoproteins. In particular, the changes of the viral hemagglutinin proteins enable the virus to avoid the immunological defence of the host organism (1). Consequently, the control of the influenza by vaccination is not completely effective. Today, only amantadine (1-adamantanamine hydrochloride) and its structural analogue, rimantadine (α-methyl-1-adamantanemethylamine hydrochloride) are though to be effective in prophylaxis and treatment of influenza infection (2). These compounds are effective only against influenza A virus infections, but not against influenza B viruses (3).

It is reported that *Sanicula* has been used as a traditional medical plant in the treatment of wounds caused by some dermatological and gastrointestinal diseases (4). This plant was previously investigated for its antimicrobial activity. These investigations indicate that saponins of *Sanicula* are responsible for its antimicrobial activity (5). So far antiviral activity of *Sanicula* extract has been determined only in experiments performed in a phage-bacteria system (6). In that system, it was found that *Sanicula* extract inhibited the adsorption of bacteriophage T2hr$^+$ on the host bacteria.

In the present study, we have examined the effects of *Sanicula europaea* L. (*Apiaceae*) leaf extracts on the multiplication of influenza viruses in Madin-Darby canine kidney (MDCK) cells.

MATERIALS AND METHODS

Cells and viruses. MDCK cells were used as host cells. These cells were grown in Eagle's minimum essential medium (EMEM) containing 10% fetal calf serum (Gibco), penicillin G (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained in a humidified athmosphere containing 5 % CO_2 at 37 °C. Influenza viruses A/PR/8/34 (H1N1), A/Victoria/1/75 (H3N2) and B/Lee/40 were used in the present experiments. The viruses were

[1] Corresponding author. Fax: 90-212-519 08 34.

The abbreviations used are: RNP, ribonucleoprotein; MOI, multiplicity of infection, pfu, plaque forming unit.

0006-291X/96 $18.00

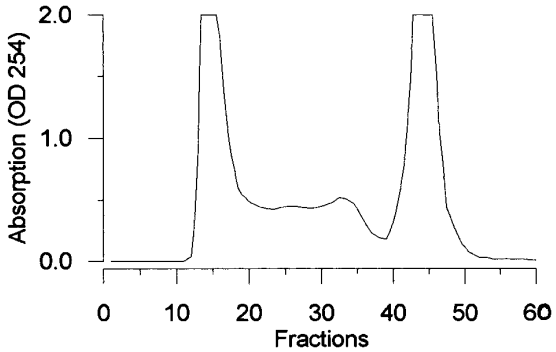

FIG. 1. The Sephadex gel filtration column chromatography of water soluble extract of *Sanicula europaea* L. leaves. Dimension of column is 2.5×100 cm. 250 mg of extracts were loaded.

grown in the allantoic cavity of 11 day-old chick embryos at 35.5 °C for 48 hours. The allantoic fluid was harvested and clarified by centrifugation at 3,000×g for 10 minutes and then the resulting supernatant was stored in small aliquots at −80 °C.

Preparation of the plant extract. Sanicula europaea L. was grown in the Botanical Garden of Department of Biology (Istanbul University) and fresh leaves of the plant were collected between October and January. Fresh leaves of *Sanicula* were extracted with bidistilled water at room temperature. The extract was filtered through cheesecloth and then clarified by centrifugation at 17,000×g for 25 minutes. The supernatant was frozen and then lyophilized to the dry powder. Crude extract dissolved in bidistilled water at the concentration of 50 mg/ml was passed through Sephadex G-100 (Pharmacia) gel filtration chromatography and separated into four fractions. Each fraction was lyophilized and used in anti-influenza virus assays.

Cell viability and cell growth test. Sanicula extract was added to MDCK cell cultures at various concentrations as indicated and the cells were incubated for 72 hours at 37 °C. After the incubation period, the cells were harvested and the number of viable cells were counted according to the trypan blue exclusion method. For the growth test, MDCK cells were seeded in 24-well plate and incubated at 37 °C in the presence or absence of *Sanicula* extracts. Each day during the log phase of cell growth, the number of viable cells was determined by the trypan blue exclusion method.

Plaque assay. For plaque inhibition assay, confluent monolayer cultures of MDCK cells in 6-well plate were washed twice with MEM and infected with influenza viruses at the appropriate multiplicity of infection (MOI). After adsorption for 30 min at 37 °C, cells were washed twice with MEM and overlayed with maintenance medium (MEM containing 0.8 % agarose, 0.2 % Bovine Serum Albumine and 4 μg/ml trypsin). The cultures were incubated at 34 °C for 2 to 3 days and plaques were visualized by staining cells with amido black.

Hemagglutination titer tests. Confluent monolayer cultures in 24-well plate were washed and infected with influenza viruses at a MOI of 0.02 pfu (plaque forming unit) per cell. After adsorption for 30 min at 37 °C, cells were washed with MEM again. Cultures were incubated in the maintenance medium (without agarose) in the presence or absence of *Sanicula* extract. Viruses present in the culture fluid were quantified by HA titer at different times.

Direct pre-infection incubation (DPI) assay. Suspensions of the virus were exposed to the 100 μg/ml concentration of extract at 37 °C for 1 hour. After then, the titer of the infectious viruses was determined by the plaque assay.

RNA synthesis in vitro. Ribonucleoprotein (RNP) cores were isolated according to the method previously reported (7,8,9). RNA synthesis was carried out as described (10).

RESULTS AND DISCUSSION

Four different fractions were obtained from the crude extract of *Sanicula europaea* L. leaves through gel filtration chromatography (Fig. 1). Separated fractions between 10 and 20, 21 and 28, 29 and 39 and, 40 and 50 were pooled and designated Fraction I, II, III and IV, respectively. The effect of each fraction on the growth of MDCK cells was tested. The growth rate (Fig. 2a) or final cell mass (Fig. 2b) of MDCK cells were not affected by Fractions I, II, or III up to the concentration of 100 μg/ml. However, in the presence of Fraction IV, the growth rate and the final cell mass of MDCK cells decreased.

The effect of *Sanicula europaea* L. extracts on the virus multiplication was determined by

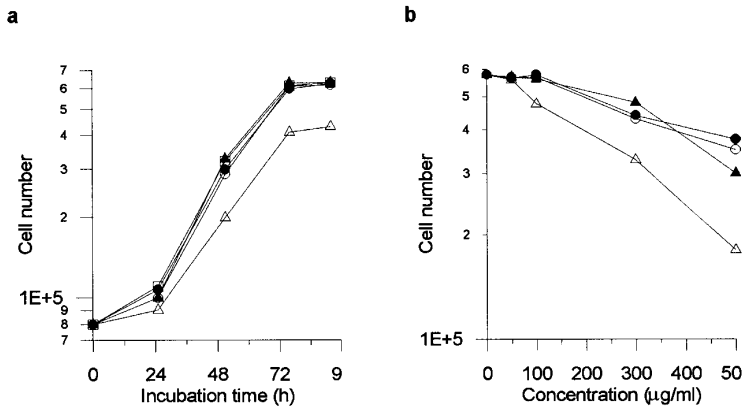

FIG. 2. Effect of *Sanicula* extracts on the growth of MDCK cells. (**a**) MDCK cells were seeded (8×10^4) in 24-well plate and incubated at 37°C in the absence (□) or presence of 100 μg/ml of *Sanicula* extracts [Fraction I (●), II (○), III (▲), or IV (△)]. After incubation for indicated periods, the viable cell number was determined. (**b**) MDCK cells were seeded (8×10^4) in 24-well plate and incubated at 37°C in the absence or presence of 50, 100, 300, or 500 μg/ml of *Sanicula* extracts. After incubation for 72 hours, the viable cell number was determined. Symbols are the same as in (**a**).

plaque inhibition test. Plaque formation by influenza A/PR/8/34 was completely inhibited by Fractions I, II and III at 100 μg/ml, while the Fraction IV was not found effective at non-taxic concentrations (up to 50 μg/ml). According to the plaque formation assay, the same extracts were not effective against influenza B/Lee/40 multiplication (Fig. 3). Influenza A/Vic/1/75 produced microscopic plaques in the presence of extracts (data not shown). The effect of Fraction III on virus multiplication was also evaluated with the HA titer test (Fig. 4). The results of the HA test are in good agreement with those of the plaque inhibition assay. In the presence of the fraction III, there was no increase in HA titer of influenza A/PR/8/34. If Fraction III is excluded from the medium after the adsorption period, influenza A/PR/8/34 gave the same HA titer as the control (Fig. 4a). This result shows that Fraction III does not interfere with adsorption of influenza viruses to the cells, but exerts its inhibitory effect on the intracellular replication process in infected cells. The growth of influenza B/Lee/40 was not affected by Fraction III in this assay (Fig.4b). On the other hand, Fractions II and III were

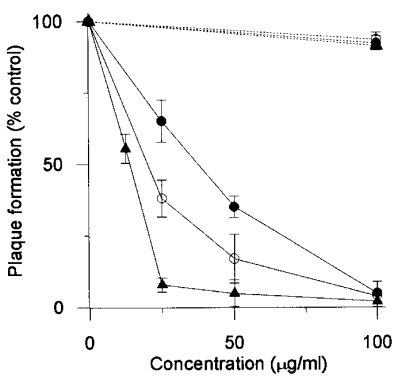

FIG. 3. Effect of *Sanicula* extracts on plaque formation. Plaque formation by influenza A/PR/8/34 (—) and influenza B/Lee/40 (· · ·) was carried out in the presence of increasing concentrations of *Sanicula* extracts [Fraction I (●), II (○), and III (▲)]. Vertical lines represent mean ± SD of 4 experiments.

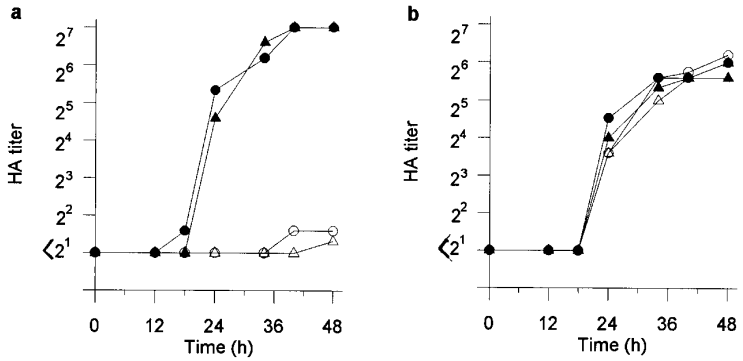

FIG. 4. Effect of Fraction III on the multiplication of influenza A/PR/8/34 (**a**) and B/Lee/40 (**b**). MDCK cell monolayers were infected at a MOI of 0.02 pfu per cell in the presence or absence of Fraction III at 100 μg/ml and incubated for 48 hours. Supernatant viruses were quantified by HA titer at different times. (●) − extract (control); (▲) + extract (at adsorption period), − extract (at replication period); (○) + extract in both adsorption and replication period; (△) − extract (at adsorption period), + extract (at replication period).

also tested for virucidal activity on influenza A/PR/8/34 but no activity was found using the DPI assay at 100 μg/ml concentration (Fig. 5).

Penetration of the influenza viruses into the cytosol occurs by an acid-activated, hemagglutinin-mediated fusion event between the viral membrane and the limiting membrane of endosome (11). At low concentration (0.1-5 μM) amantadine exerts its antiviral activity at the period of uncoating and at the period of virus assembly (12, 13). The high and clinically anattainable concentrations (>0.1 mM) of amantadine results in raising the pH of intracellular vesicles, and non-specifically inhibits other viruses which enter cells by endocytosis, such as influenza B, rubella and certain paramyxoviruses (14). Some weak basic molecules such as methylamine, chloroquine and ammonium chloride can also inhibit the uncoating of influenza viruses non-specifically by increasing endosomal pH (15). In contrast, *Sanicula* extracts specifically inhibited influenza A viruses (Fig. 3). Influenza B/Lee/40 was not affected by the same extracts.

In order to understand the mechanism of inhibition, the effects of *Sanicula* extracts under different experimental conditions were studied. It was found that these antiviral effects could neither be attributed to direct inactivation of the influenza viruses nor by inhibition of their adsorption to the host cells. It seems likely that *Sanicula* extracts exert their inhibitory effect on the intracellular replication process in the infected cells. Preliminary experiments showing that the RNA polymerase activity of influenza A virus measured in vitro is inhibited by the

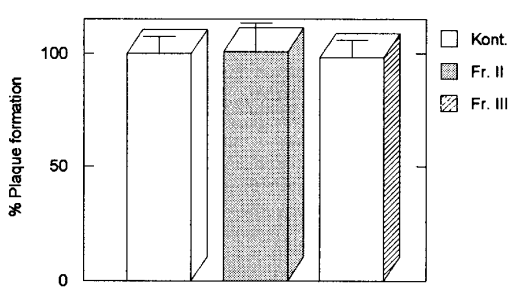

FIG. 5. Preinfection treatment of Fractions II and III at 100 μg/ml concentration on influenza A/PR/8/34. Vertical lines represent ±SD of 3 experiments.

addition of extracts (data not shown) point to such a possibility, although at the present it has not been tested whether the RNA polymerase activity of influenza B viruses is affected.

Since the inhibitory mechanism of *Sanicula* extracts on multiplication of influenza A viruses is unclear, further invastigation is necessary. The mechanism of action of *Sanicula* extracts and the nature of active molecule(s) remains to be studied.

ACKNOWLEDGMENTS

This work was supported by the Research Fund of the University of Istanbul, Project No. 551/121192 (K. Turan, A. Kuru) and in part by a grant for Biodesign Research Program from RIKEN (K. Nagata).

REFERENCES

1. Webster, R. G., Laver, W. G., Air, G. M., and Schild, G. C. (1982) *Nature* **296,** 115–121.
2. Tominack, R. L., and Hayden, F. G. (1987) *Infect. Dis. Clin. North America* **1,** 450–478.
3. Reines, E. D., and Gross, P. A. (1988) *Med. Clin. North America* **72,** 691–715.
4. Winkelmann, W. (1951) Die Wirkstoffe Unserer Heilpflanzen Darstellung und Anwendung, pp. 131–134, Verlag Otto Walter AG Olten.
5. Trezenschik, U., Przyborowski, R., Hiller, K., and Linzer, B. (1967) *Pharmazie* **22**(12), 715–717.
6. Turan, K., and Kuru, A. (1996) *Tr. J. Biology* **20**(3), 225–234.
7. Gregoriades, A. (1973) *Virology* **54,** 369–383.
8. Honda, A., Uéda, K., Nagata, K., and Ishihama, A. (1987) *J. Biochem.* **102,** 1241–1249.
9. Honda, A., Uéda, K., Nagata, K., and Ishihama, A. (1988) *J. Biochem.* **104,** 1021–1026.
10. Nagata, K., Sakagami, H., Harada, H., Nonoyama, M., Ishihama, A., and Konno, K. (1990) *Antiviral Res.* **13,** 11–22.
11. Marsh, M., and Helenius, A. (1989) *Adv. Virus Res.* **36,** 107–151.
12. Kato, N., and Eggers, H. J. (1969) *Virology* **37,** 632–641.
13. Hay, A. J., Zambon, M. C., Wolstenholme, A. J., Skehel, J. J., and Smith, M. H. (1986) *J. Antimicrob. Chemother.* **18,** suppl. B, 19–29.
14. Couch, R. B., and Six, H. R. (1986) *in* Antiviral Chemotherapy: New Directions for Clinical Application and Research (Mills, J., and Corey, L., Eds.), pp. 50–57, Elsevier, New York.
15. Hay, A. J. (1986) *Chemica Scripta* **26,** 77–81.