Anti-HIV activity of alkaline extract from pine seed shells 
(Pinus koraiensis)

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The alkaline extracts from pine seed shells (Pinus koraiensis) suppressed the human immunodeficiency virus (HIV)-induced cytopathicity using HIV (HTLV-III) infected MT-4 cells in vitro, and showed extremely low cytotoxicity. The active substances were acid polysaccharides containing uronic acids. No animal died and no harmful effect was observed at a concentration of 1.05 g per kg body weight. We also studied the clinical effects of alkaline extracts on the protection of feline immunodeficiency virus (FIV) infection. Protection against infection by FIV was achieved by oral administration of the alkaline extracts with usual food.

Key words: alkaline extract, pine seed shells, feline immunodeficiency virus, cytopathogenicity.

Introduction
It is important to find effective chemotherapy for acquired immunodeficiency syndrome (AIDS). Some antiviral agents have been licensed for use in humans.1,2 Long-term suppression chemotherapy appears to improve survival in patients with AIDS; unfortunately, long-term chemotherapy causes significant side effects.

Pine cone extracts show immunopotentiating activity,3,4 antimicrobial activity5 and antiviral activity.6 Leaves of pine are famous as a Kanpo medicine. Pine seeds were used as preserved foods from thousands of years ago, and as health foods in China and Korea from older times. Recently, we reported that alkaline extracts from Rooibos tea leaves containing sprigs (Aspalathus linearis) showed a potent anti-HIV activity, but low cytotoxicity.7,8 The active substances were acid polysaccharides.

In this paper, we describe the isolation of alkaline extracts from pine seed shells, and clinical effects of the alkaline extracts on feline immunodeficiency virus (FIV).

Materials and methods
Pine seed shells (Pinus koraiensis) free from oil were obtained from Pine Nuts Co., Nagoya, Japan. Four-week-old mice (Balb/c strain, female; SPF) were obtained from Japan SLC Inc., Hamamatsu, Japan. The animals were kept with standard food and water ad libitum under a 12-hour light–dark cycle (lights on 0700). Cats were obtained from a local breeder and were kept at the animal facilities of Sunsho Co., Hiroshima, Japan, and Aichi Medical University. The animal experiments were performed within the guidelines of Ethics Committee for animal care.

Preparation of alkaline extracts
Pine seed shells were extracted twice with hot water (100-fold volume of the shells) at 85°C for 3 h. The pine seed shells were further extracted with 1% sodium hydroxide (10-fold volume of the shells) at 45°C for 3 h. The alkaline extract was filtered through two layers of gauze, and designated as the ‘crude extract’.

Assay method for anti-HIV activity in vitro
Flat bottom, 96-well plastic microtiter trays (Falcon, Becton Dickinson, CA, USA) were filled with 100 µL of complete medium and added test substances. Anti-HIV activity was assayed by the method of Pauwels et al.9 and Nakashima et al.10 Briefly, HTLV-IIIIB infected MT-4 cells and non-infected cells were spread in a plate with the test substances, and incubated for 4 days. After incubation, live cells were detected using the MTT (tetrazolium) method. Anti-virus activity was expressed as the 50% protection activity of infected cells by the test substances (EC50; effective concentration). The cytotoxic activity of the test substances was expressed as the cytotoxic concentration of test substances with 50% cell damages (CC50; cytotoxic concentration).

Column chromatography
Alkaline crude extracts were precipitated with ethanol, and the precipitated fraction (25–75% ethanol precipitated fraction) was applied on a Cellulose G–700m column (1.6 × 50 cm) which was previously equilibrated with distilled water. The elution was performed with distilled water, and each fraction was 2.5 mL.

Analytical methods
Neutral sugar content and uronic acid content were measured

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by the phenol–sulfuric acid method,\textsuperscript{11} and the carbazole
method,\textsuperscript{12} respectively.

\textbf{Acute virulent test}

A given concentration of crude alkaline extract (1.05 g/kg
body weight) from pine seed shells was injected into stomach
by catheter. After administration, behaviour, number of dead
animals, and body weight change were checked. After 5 days,
animals were killed and macroscopic observation of organs
was undertaken.

\textbf{Administration of crude alkaline extract from pine seed
shells}

\textit{Experiment 1:}

Thirty-day-old kittens (mixed breed, both sexes) were
obtained from a local breeder, and kept with foods containing
the crude alkaline extracts (30 mg) for 30 days. Dry powder
of the extracts was sprinkled over the food and mixed. After
this period, 0.5 mL of whole blood from FIV-infected cats
was injected intravenously. After intravenous injection of
FIV, the animals were kept for a further 2 months, and the
FIV-positivity was examined.

\textit{Experiment 2:}

Adult cats of mixed breed (1–3 years old, both sexes) were
obtained from a local breeder, and kept with foods containing
3 g pine seed shell powder which contains approximately 2%
of the active substances for 1–2 months. Pine seed shell pow-
dder was sprinkled over the food and mixed. After this period,
1.0 mL of whole blood from FIV-infected cats was injected
intravenously. After the intravenous injection of FIV-positive
whole blood, the animals were kept for a further 4 months.
During further feeding for 4 months, FIV-positive blood
was injected twice at 1 month intervals and FIV-positivity
was examined for every month.

\textbf{Results}

\textbf{In vitro experiments}

Alkaline extracts from pine seed shells suppressed the human
immunodeficiency virus (HIV)-induced cytopathicity using
HIV (HTLV-III) infected MT-4 cells \textit{in vitro}; its 50\% effective
concentration (EC\textsubscript{50}) was 176 µg/mL, while the 50\%
cytotoxic concentration (CC\textsubscript{50}) was > 0.9 mg/mL (Fig. 1). As
shown in Fig. 2, the crude extracts from pine seed shells
showed several peaks in optical density at 230 nm, and two
peaks in neutral sugars and uronic acids on a Cellulofine GC-
700 column chromatography. Neutral sugar content in the
first and the second peaks were almost identical. Uronic acid
contents in the first and second peaks were 263 µg/mL (glu-
curonic acid as the standard) and 141 µg/mL, respectively.
Anti-HIV activity in the first peak was higher than that in the
second.

We examined sugar components in the extracts. The
extracts contained 27\% of reducing sugar, 22\% of neutral
sugars and 19\% of uronic acid.

\textbf{In Vivo experiments}

We examined the acute effects of extracts on the mouse using
oral administration. No animal died and no harmful effect
was observed with the crude extracts at a concentration of
1.05 g per kg body weight (Fig. 3).

\textbf{Discussion}

Alkaline extracts from pine seed shells appear to have anti-
HIV activity. Cytotoxic activity was not observed at a con-
centration lower than 0.9 mg/mL. Alkaline extracts were
partially purified with column chromatography using Cel-
lulofine GC-700. The active material contains relatively high
amounts of uronic acid. The substances were acid polysac-
charides containing a carboxyl group (COOH). In general, a
carboxyl group easily reacts with phenolic compounds,
which are rich in plant materials. It is probable that the alka-
line extracts used in this study would be a complex of acid polysaccharides and phenolic compounds.

2’,3’-Dideoxynucleosides including 3’-azido-3’-deoxythymidine (AZT) act as potent inhibitors of reverse transcriptase after intracellular phosphorylation. Inhibitors of reverse transcriptase such as AZT and 2’,3’-dideoxyinosine are now available for the treatment of AIDS and the AIDS-related complex. Long-term suppressive chemotherapy appears to improve survival in patients with AIDS. However, these agents show severe side effects in long-term chemotherapy. The treatment of AIDS patients and HIV infectants may include a daily intake of foods or agents which have no or extremely low side effects, but which suppress the infectant and related illness. Because HIV is a retro-virus and goes into cellular DNA, it is almost impossible to kill the virus when a retrovirus goes into cellular DNA. It may be necessary to live with the virus as a carrier. For these reasons we tried to isolate active substances with a high LD<sub>50</sub> and to use them clinically as anti-viral foods.

We used cats as experimental animals for clinical in vivo experiments. According to the in vitro experiments, pine cone extracts protected against the infection by herpes simplex virus and influenza virus only when administered simultaneously. Therefore, we fed the pine seed extracts before injection of FIV for 2–4 weeks. Table 1 shows that the extracts from pine seed shells protected against infection of FIV with oral administration. Both kittens and adult cats were given foods with active substances before and after infection. Fukuchi et al. reported that pine cone extracts inhibited Herpes simplex virus (types 1 and 2 strains), and that the protection was attributable to inhibition of virus adsorption. Unten et al. have reported that the extracts from pine seed shells protect against infection with influenza virus in vitro. Other high molecular weight substances of plant origin and sulfated polysaccharides show anti-HIV activity, and protect against virus adsorption. It is questionable whether high molecular weight substances can reach target cells when consumed as foods. Recently, we reported that acid polysaccharide and oligosaccharides from Rooibos tea leaves containing sprigs (Aspalathus linearis) show anti-HIV activity and have low cytotoxicity. We have also found that oligosaccharides from pine seed shells show anti-HIV activity in vitro (M. Nakano et al., unpubl. data). These results suggest that acid polysaccharide from pine seed shells may protect against HIV infection through virus adsorption.

Acknowledgement. The authors express their thanks to Mr Masazumi Yoshihara, Sunsho Co., for his kind help with the in vivo FIV experiments.
Anti-HIV activity of alkaline extract from pine seed shells (Pinus koraiensis)
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松子殼（Pinus Koraiensis）醤性抷提物的抗人類免疫缺陷病毒（HIV）的活性

摘要

作者在體外用HIV（HTLV-III）感染MT-4Cells，然後觀察松子殼（Pinus Koraiensis）齦性抷提物對HIV引起細胞病理的抑製作用。他們發現該抷提物的細胞毒性極低，其活性物質是含有尿酸的酸性多糖，每公斤體重給予1.05克時，沒有動物死亡和傷害。

他們也研究了齦性抷提物對貓免疫缺陷病毒（FIV）感染的臨床效果，結果發現齦性抷提物與一般食物同時口服可獲得保護作用。

References