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 activity[5] 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-IIIB 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 (EC_{50}; effective concentration). The cytotoxic activity of the test substances was expressed as the cytotoxic concentration of text substances with 50% cell damages (CC_{50}; cytotoxic concentration).

Column chromatography

Alkaline crude extracts were precipitated with ethanol, and the precipitated fraction (25–75% ethanol precipitated fraction) was applied on a Cellulofine GC-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–sulfulic acid method,[11] and the carbazole method,[12] respectively.

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.

Administration of crude alkaline extract from pine seed shells

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.

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 powder 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.

Results

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 *in vitro*; its 50% effective concentration (EC_{50}) was 176 µg/mL, while the 50% cytotoxic concentration (CC_{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 (glucuronic 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.

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).

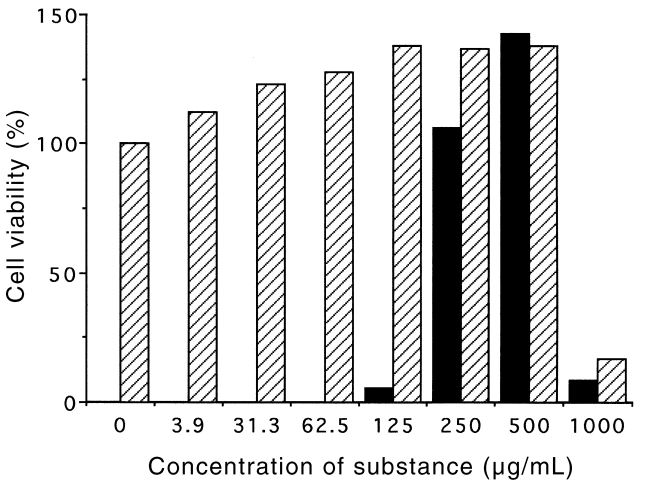

Figure 1. Protection of HIV-induced cytopathicity with alkaline extract from pine seed shells *in vitro*. The human T lymphotropic virus type 1 (HTLV-1) positive T-cell line, MT-4 and HTLV-1 non-infected T-cell line, MOLT-4 were used. ■, Inf-MT-4; HIV-1 infected MT-4 cells: ⍁, Mock MT-4; non-infected MOLT-4 cells (control).

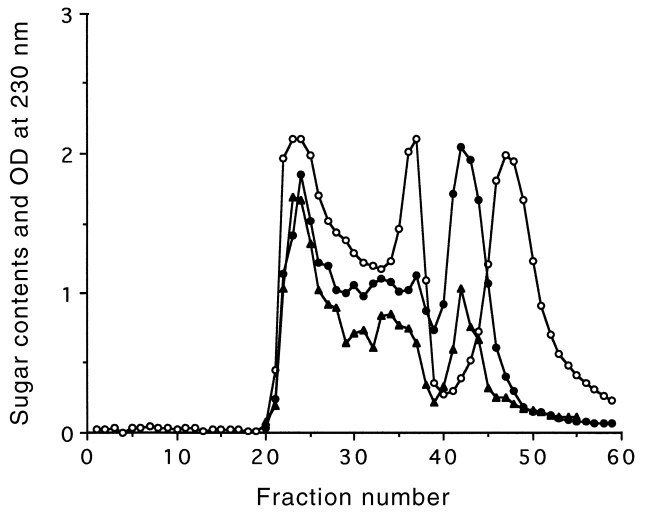

Figure 2. Column chromatography of alkaline extract from pine seed shells. ○, optical density at 230 nm; ●, neutral sugar content; ▲, uronic acid content.

For the *in vivo* experiments, we decided on a daily intake of alkaline extracts which was 500–1000-fold the EC_{50} in *in vitro* experiments. As shown in Table 1, 80% (4/5) of animals in experiment 1 and 75% (3/4) of animals in experiment 2 were FIV-negative. In the control group, however, all animals (n = 3) became FIV-positive.

Discussion

Alkaline extracts from pine seed shells appear to have anti-HIV activity. Cytotoxic activity was not observed at a concentration lower than 0.9 mg/mL. Alkaline extracts were partially purified with column chromatography using Cellulofine GC-700. The active material contains relatively high amounts of uronic acid. The substances were acid polysaccharides 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-

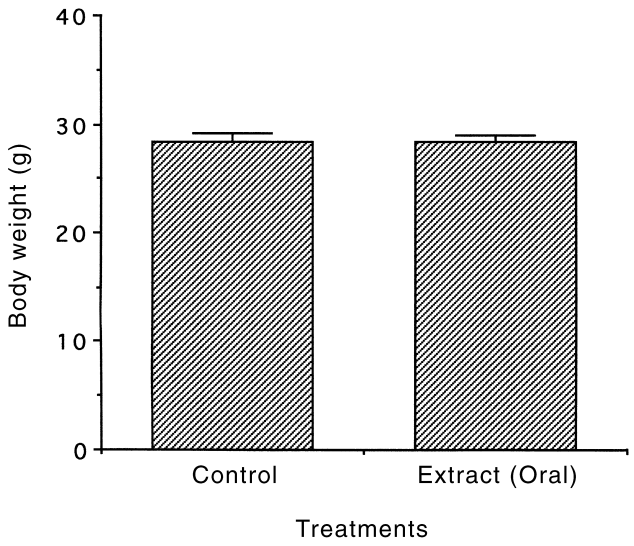

Figure 3. Acute virulent test of crude alkaline extract from pine seed shells. After oral administration of crude alkaline extract of pine seed shells, animals (Balb/c mouse) were fed for 5 days.

Table 1. Protective effects of alkaline extracts from pine seed shells on feline immunodeficiency virus infection

	Treatments	Infection (%)
Experiment 1	With extract (n = 6)	17 (1/6)
	Control (n = 4)	100 (4/4)
Experiment 2	With extract (n = 4)	25 (1/4)
	Control (n = 4)	100 (4/4)

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.[13,14] 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.[15,16] 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_{50}, 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[6] and influenza virus[17] 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.[6] Unten *et al*.[4] 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.[18–23] 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 protect against virus adsorption, and have low cytotoxicity.[7,8,24,25] 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.

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松子殼（ Pinus Koraiensis ）鹼性抽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 ）鹼性抽提物對 HIV 引起細胞病理的抑製作用。他們發現該抽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。

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

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