[Jpn. J. Parasitol., Vol. 39, No. 2, 220–225, April, 1990]

Lethal efficacy of leaf extract from *Perilla frutescens* (traditional Chinese medicine) or perillaldehyde on *Anisakis* larvae *in vitro*

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(Accepted for publication; April 13, 1990)

Abstract

The authors previously reported that a saline (0.9%) extract from *Perilla frutescens,* used as a traditional Chinese medicine, effectively destroyed *Anisakis* larva *in vitro.* In this report, we analyzed the effective components of perilla leaves. Because active compound was extracted from saline into the ether fraction, methanol extracts were fractionated after first being treated with HCl at pH 3, then with $NaHCO_3$ at pH 10 (fraction 1). In general, this fraction was rich in phenol, ketone or aldehyde groups. Perillaldehyde, a known component of perilla leaves, was detected (using gas chromatography) in this fraction. A dose-dependent lethal efficacy was observed only in fraction 1. Authentic perillaldehyde could kill *Anisakis* larvae at a minimum effective dose of 125 μg/ml. Perillyl alcohol had a lethal effect, too, however, it was not detected in any fractions of the extract. Based on these results, it was suggested that perillaldehyde is the most important active component for killing *Anisakis* larva in an extract of *P. frutescens.* Neither pyrantel pamoate nor thiabendazole had any lethal effect even at a concentration of 1 mg/ml.

In the saline solution containing perillaldehyde (125 μg/ml), more than 90% of larvae lost spontaneous movement within 8 hr, however total killing of the worm was not observed earlier than 24 hr.

Key words: *Anisakis, Perilla frutescens,* perillaldehyde, perillyl alcohol

Introduction

Anisakiasis was first reported in the Netherlands in 1960 (Van Thiel *et al.*). This disease was initiated by the invasion of *Anisakis* larvae into the human digestive tract when infected raw sea-fish were ingested. Since the Japanese diet consists of the ingestion of raw fish, anisakiasis became a prominent parasitic disease. So far, 4,682 cases have been reported in Japan (Ishikura *et al.*, 1988). Recently, the number of cases of the disease in the United States has increased markedly, too (Schantz, 1989).

Until recently, the only known effective therapy for this disease has been removal of the larvae. Then, the authors investigated various substances *in vitro* in an attempt to discover an effective therapeutic drug, and it was reported that a saline extract from *Perilla frutescens* Britton var. *acuta* Kudo f. *viridis* Makino was effective in destroying *Anisakis* larvae (Kasuya *et al.*, 1988). *P. frutescens* had been eaten along with raw fishes as well as used as a traditional Chinese medicine because of its sedative or antidotic actions (Honda *et al.*, 1986). This report was an attempt to isolate the active component(s) having antinematodal effects *in vitro* using *Anisakis* larvae.

Materials and Methods

Anisakis larvae

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Anisakis larvae (type I, the 3rd stage) were separated from the internal organs of fresh pollacks (*Theragra chalcogramma*) as described by others (Tanaka and Torisu, 1978).

Reagents

Perillaldehyde and perillyl alcohol were purchased from Katayama Chemical (Osaka, Japan) and Aldrich Chemical Company, Inc. (Milwaukee, WIS, USA), respectively. Pyrantel pamoate powder was supplied from Pfizer (Tokyo, Japan), and thiabendazole (Merck, Darmstadt, FRG) was milled from a tablet. Other reagents were purchased from Katayama Chemical.

Extracts from perilla

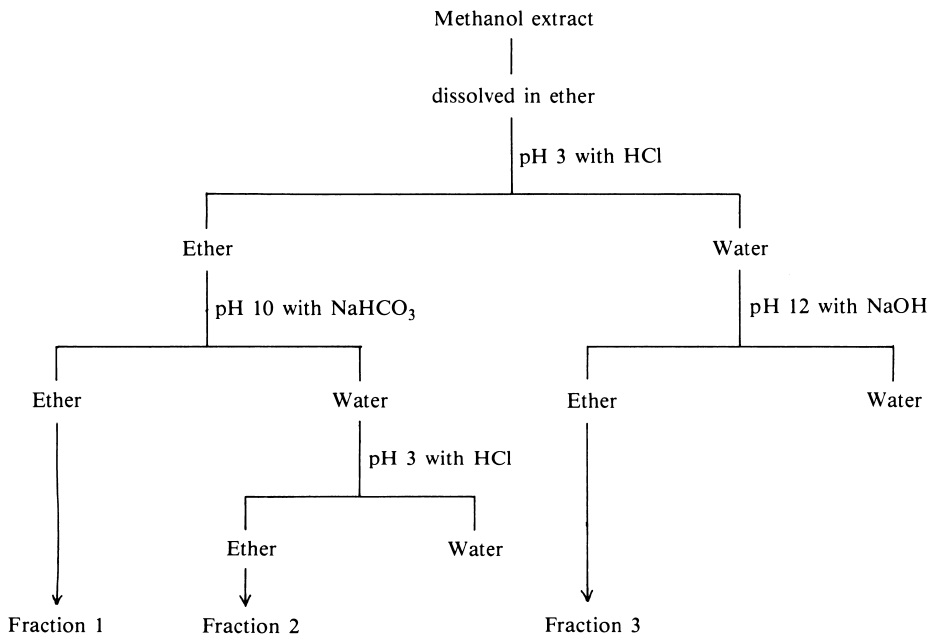
Leaves of *Perilla frutescens* Britton var. *acuta* f. *viridis* Makino were washed using a synthetic detergent for vegetables (Kao, Tokyo, Japan), rinsed 3 times with water, washed once with distilled water, then freeze-dried. Saline (0.9%) extract was prepared as follows: dried leaves were homogenized with a glass homogenizer at a final concentration of 5% (w/v) for 5 min, then extracted overnight at 4°C; supernatant, after centrifugation at 9000 g for 30 min, was used; and, for fractionation, 1.5 g of dried leaves were extracted with 200 ml of methanol for 3 days at room temperature (25°C) then filtered through a paper filter (No. 2, Advantec, Tokyo, Japan).

Fractionation of extracts

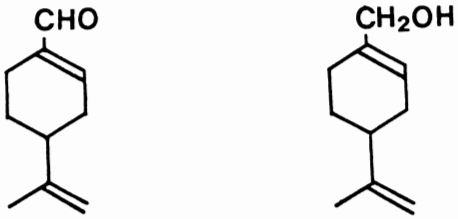
The saline extract first was mixed and shaken with ethyl ether, and then the lethal efficacy of the saline fraction was assayed. Methanol extract was dried using a rotary evaporator (Shibata EL 131, Tokyo, Japan) then redissolved in ether (150 ml). Substances in ether were mixed with 3N HCl to pH3 (NaCl was added to disperse emulsion) then shaked in separatory funnel. Both ether and water fractions were fractionated further according to the Scheme 1, as described by others (Ando *et al.*, 1967). Each fraction was dissolved in 1 ml of ethanol after evaporating the ether, then assayed for lethal efficacy and concentration of perillaldehyde and perillyl alcohol (Fig. 1).

Assay of lethal efficacy

A two-fold serial dilution of each material or fraction was made with ethanol. Twenty



Scheme 1. Fractionation of methanol extract from leaves of *Perilla frutescens*



(1) (2)

Fig. 1. Chemical structure of perillaldehyde (1) and perillyl alcohol (2).

microliters of each ethanol-based solution was dissolved into 1.98 ml of saline (0.9%). Control was maintained with a saline solution containing 1% ethanol. Eight to 12 larvae were placed into a polycarbonate tube (2 ml, Greiner, FRG) containing a prepared testing solution and incubated at 37°C. Worms were transferred into saline at room temperature after incubation (mainly for 24 hr). Loss of spontaneous movement over a 3 min period was assessed immediately after culture termination. A worm was defined as being dead after coming to a complete standstill 4 or 5 hr later when stimulated with tweezers.

Chemical analysis

Perillaldehyde and perillyl alcohol in each fraction were identified by gas chromatography (GC) and then compared with authentic samples. GC analysis was carried out using a stainless column (3 mm × 3 m) packed with PEG-6000 chromosorb W 17% (30–60 mesh) under the following conditions: injection port temp. 250°C; column temp. 170°C; carrier gas, N_2 (0.8kg/cm^2); detector, FID. Peak of perillaldehyde was detected on the chromatogram at RR_t 0.31 relative to the peak of thymol ($RR_t = 1$) used as an internal standard.

Microscopical examination

Larvae incubated with perillaldehyde solution (125 μg/ml) for 24 hr were fixed with a 10% formalin solution, embedded in paraffin, and transversally cuted into thin sections. Hematoxylin and eosin-stained preparations were examined by microscope.

Results

Larvae of *Anisakis* incubated in saline extract (5% w/v) for 24 hr completely lost their motility even when stimulated with tweezers 4–5 hr after being transferred into saline. In some experiments, larvae were kept in saline for 5 days or more, and no worms assessed as dead recovered, validating our definition. The lethal

Table 1. Lethal efficacy of each fraction of *P. frutescens* on *Anisakis* larvae *in vitro*

Fraction	% death and % loss of spontaneous movement (in parenthesis) of worms*			
Concentration†	1%	0.5%	0.25%	0.125%
1	100 (100)	64.6 (100)	0 (65.2)	0 (14.3)
2	0 (25.0)	0 (0)	not done	not done
3	0 (0)	not done	not done	not done
ethanol	0 (0)	not done	not done	not done

* Worms were incubated at 37°C for 24 hr. Mean of the duplicate fractionations.
† Each fraction was dissolved and diluted with ethanol then added to saline at a concentration of 1% (v/v).

efficacy of the saline extract was completely counteracted (100% to 0%) by mixing and shaking with ether, leading the authors to analyze ether-fractions.

All larvae were killed in a 1% solution of fraction 1. A dose-dependent lethal efficacy was seen. All the other fractions including a 1% ethanol solution had no lethal efficacy (Table 1). Almost all perillaldehyde was detected in fraction 1. Perillyl alcohol was not detected in any of the fractions (Table 2).

All worms were destroyed with authentic compounds at a concentration of more than 125 μg/ml of perillaldehyde. Partial destruction or loss of spontaneous movement was observed at a concentration of 31.25 μg/ml. The lethal efficacy of perillyl alcohol was slightly lower than perillaldehyde (Table 3). Contrarily, pyrantel pamoate and thiabendazole, known antinematodal drugs, had no lethal effect on *Anisakis* larvae at the concentration of 1 mg/ml.

A time course of the mortality and % loss of spontaneous movements were studied in the culture containing 125 μg/ml of perillaldehyde at 37°C. Approximately 50% of the worms stopped their spontaneous movements within 4 hr, however, no dead worms were observed at this time. More than 90% of the worms lost spontaneous movements within 8 hr, and a few larvae had began to die. Total destruction of *Anisakis* larvae occurred within 24 hr (Fig. 2).

Microscopical examinations of dead larvae demonstrated no particular change.

Table 2. Concentration of perillaldehyde and perillyl alcohol in each fraction

Fraction	Concentrations (mg/ml)*	
	perillaldehyde	perillyl alcohol
1	7.3	not detected
2	0.1	not detected
3	not detected	not detected

* Dried leaves (1.5 g) were extracted, fractionated then dissolved in 1 ml of ethanol. Mean of the duplicate fractionations.

Discussion

Perilla leaves have been used as traditional Chinese medicine and several extracted components, including perillaldehyde and perillyl alcohol, have been reported (Ito, 1964). Pharmacological characteristics of perillaldehyde or other contents in perilla have been identified as having antisedative (Sugaya *et al.*, 1981), antiallergic (Nikaido *et al.*, 1981), antibacterial (Okazaki *et al.*, 1951), and antifungal properties (Honda *et al.*, 1984). Our study has identified a heretofore unknown property of perillaldehyde

Table 3. Dose dependent effects of perillaldehyde or perillyl alcohol on *Anisakis* larvae *in vitro*

Drug \ Dose (μg/ml)†	% death and % loss of spontaneous movement (in parenthesis) of worms*				
	250	125	62.5	31.25	15.625
Perillaldehyde	100 ± 0 (100 ± 0)	100 ± 0 (100 ± 0)	42.9 ± 29.7 (65.1 ± 13.7)	27.0 ± 20.3 (35.3 ± 16.0)	0 ± 0 0 ± 0
Perillyl alcohol	100 ± 0 (100 ± 0)	95.8 ± 4.2 (100 ± 0)	33.3 ± 33.3 (62.5 ± 37.5)	0 ± 0 (0 ± 0)	not done

* Worms were incubated with each solution at 37°C for 24 hr. Mean ± SEM of 3 experiments.

† Compounds were dissolved in ethanol then added to saline at a concentration of 1% (v/v).

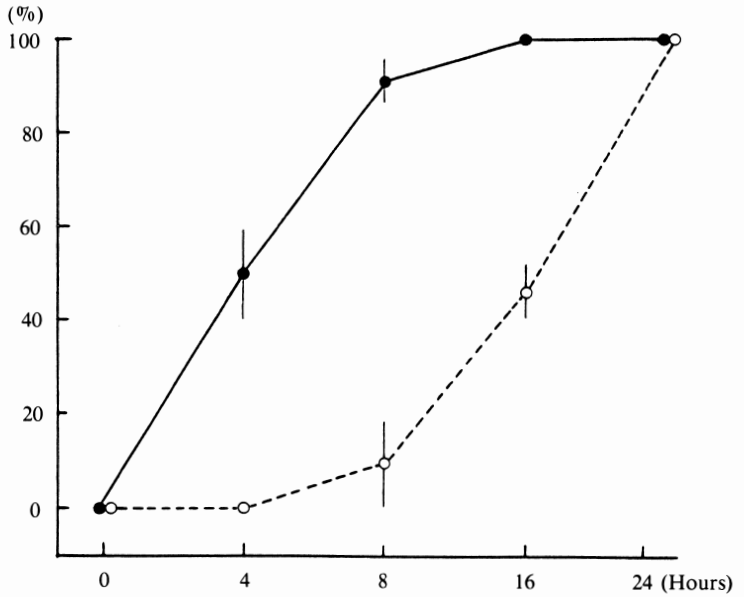


Fig. 2. Time course of loss of spontaneous movements (●—●) and mortality (○--○) of *Anisakis* larva by perillaldehyde (125 μg/ml). Mean ±SEM of 3 experiments.

or perillyl alcohol, i.e., an antinematodal efficacy when used on *Anisakis* larvae *in vitro*. Perilla has been used as a treatment for "toxicosis caused by fish meat" (Hino and Isshiki, 1926), but the definition of "toxicosis" is not clear, which might mean that anisakiasis could be lumped together with allergic reactions or bacterial infections brought about by the ingestion of fish meat.

Only fraction 1 exhibited any lethal efficacy and contained the most amount of perillaldehyde (Table 1 and 2). In addition, an authentic compound of perillaldehyde destroyed the larva (Table 3). These evidences led the authors to hypothesize that perillaldehyde was the compound causing such a lethal efficacy in perilla. Although minimum effective dose of authentic perillaldehyde was 125 μg/ml, that of fraction 1 was 1%, i.e., 73 μg/ml. Therefore, actual concentration of perillaldehyde in fraction 1 was too low to exhibit complete killing of larvae. These results suggested further components in this fraction which had or assisted a lethal efficacy, because this fraction contained phenols, ketons and aldehydes. Perillyl alcohol was demonstrated as having a lethal efficacy (Table 3). However, it was not detected in our experimental system (Table 2).

Today, there are nematodal infections, e.g., anisakiasis and visceral larva migrans which are resistant to antinematodal drugs. Our *in vitro* assay shows 125 μg/ml of perillaldehyde totally destroys *Anisakis* larvae within 24 hr, while a 1 mg/ml solution of pyrantel pamoate and thiabendazole exhibit no lethal efficacy. If perillaldehyde kills *Anisakis* larvae *in vitro,* it is possible this compound may be applicable *in vivo,* especially since it is believed to be non-toxic in mice. It has been reported that the LD_{50} value of perillaldehyde on mice is greater than 1 g/kg of body weight (*p.o*) (Honda *et al.*, 1986). Therefore, further elucidation of the antinematodal spectrum of perillaldehyde is called for.

The killing mechanism is not clear at present. Spontaneous movements of worms are attenuated early, however, it requires 24 hr to completely kill them (Fig. 2). In addition, no histological changes can be observed. Based on this data and reports by others, some disorders in nervous system of larvae are suggestive. Perillaldehyde has been reported to prolong sleep

induced by hexobarbital-Na in mice and to inhibit the laryngeal reflex of the cat and excitation of the membranes of the snail's neurons as well as the frog's sciatic nerve fibers (Sugaya *et al.*, 1981).

Acknowledgement

We wish to thank members in Tsumura's lab. for measuring concentrations of perillaldehyde and perillyl alcohol in each fraction. Pyrantel pamoate was kindly supplied from Pfizer (Tokyo, Japan).

References

1) Ando, T., Terayama, H., Nishizawa, K. and Yamakawa, T. (1967): Methods for biochemistry research I. Asakura Syoten, Tokyo, 11–14 (in Japanese).
2) Hino, I. and Isshiki, N. (1926): Wakanyakubutsugaku, 1st ed., Dohsaigosyobo, Osaka, 23–24 (in Japanese).
3) Honda, G., Koga, K., Koezuka, Y. and Tabata, M. (1984): Antidermatophytic compounds of *Perilla frutescens* Britton var. *crispa* Decne. Shoyakugaku Zasshi, 38, 127–130.
4) Honda, G., Koezuka, Y., Kamisako, W. and Tabata, M. (1986): Isolation of sedative principles from *Perilla frutescens*. Chem. Pharm. Bull., 34, 1672–1677.
5) Ishikura, H., Kobayashi, Y., Miyamoto, K., Yagi, K., Nakajima, O., Fujita, O., Oikawa, Y., Maejima, J., Aji, T., Akao, Y. and Hayasaka, H. (1988): Transition of occurrence of anisakiasis and its paratenic host fishes in Japan, with pathogenesis of anisakiasis. Hokkaido J. Med. Sci., 63, 376–391.
6) Ito, H. (1964): Studies on the folium perillae. II. On isoegomaketone. Yakugaku Zasshi, 84, 1123–1125.
7) Kasuya, S., Goto, C. and Ohtomo, H. (1988): Studies on prophylaxis against Anisakiasis – A screening of killing effects of extracts from foods on the larva. Jpn. Assoc. Inf. Dis., 62, 1152–1156.
8) Nikaido, T., Ohmoto, T., Noguchi, H., Kinoshita, T., Saitoh, H. and Sankawa, U. (1981): Inhibitors of cyclic AMP phosphodiesterase in medicinal plants. Planta Medica, 43, 18–23.
9) Okazaki, K., Kato, H. and Wakatabe, T. (1951): Antibacterial activity of higher plants. Yakugaku Zasshi, 71, 1–5.
10) Schantz, P. M. (1989): The dangers of eating raw fish. N. Engl. J. Med., 320, 1143–1145.
11) Sugaya, A., Tsuda, T. and Obuchi, T. (1981): Pharmacological studies of Perillae Herba. I. Neuropharmacological action of water extract and perillaldehyde. Yakugaku Zasshi, 101, 642–648.
12) Tanaka, J. and Torisu, M. (1978): Anisakis and eosinophil. I. Detection of a soluble factor selectively chemotactic for eosinophils in the extract from *Anisakis larvae*. J. Immunol., 120, 745–749.
13) Van Thiel, P. H., Kuipers, F. C. and Roskam, R. T. (1960): A nematode parasitic to herring, causing acute abdominal syndromes in man. Trop. Geogr. Med., 2, 97–113.