Inhibitory Effect of Plant Extracts on Production of Verotoxin by Enterohemorrhagic \textit{Escherichia coli} O157 : H7

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The inhibitory effect of several plant extracts on the production of verotoxin by enterohemorrhagic \textit{Escherichia coli} O157 : H7 (EHEC) was investigated. The extracts from four plant species, \textit{Limonium californicum} (Boiss.) A. Heller, \textit{Cupressus lustiana} Miller, \textit{Salvia urica} Epling and \textit{Jussiaea peruviana} L., were effective on the inhibition for verotoxin production (31.3–125 \textmu g/ml). The inhibition against verotoxin production was observed at a concentration lower than the minimal inhibitory concentration (MIC) of each extract of test plants (1000 \textmu g/ml), indicating that these plant extracts would preferentially prevent the production of verotoxin rather than bactericidal effect on EHEC. These findings suggest that the administration of any appropriate plant extract might prevent the production of verotoxin on EHEC in the human intestines.

\textbf{Key words} —— plants extract, enterohemorrhagic \textit{E. coli} O157 : H7, inhibitory effect of verotoxin production

\section*{INTRODUCTION}

Enterohemorrhagic \textit{Escherichia coli} O157 : H7 (EHEC) is a food-borne human pathogen and outbreaks and sporadic cases due to infection with EHEC have been frequently reported in various places around the world.\textsuperscript{1,2} In Japan during 1996, numerous large outbreaks and sporadic cases of EHEC occurred and over 9000 individuals was affected.\textsuperscript{3} Signs and symptoms of illness by EHEC include diarrhea, hemorrhagic colitis, severe abdominal cramps, and vomiting. A significant number of cases subsequently develop hemolytic uremic syndrome (HUS), which is probably the most common cause of renal failure in children, and occasionally resulting in long-term complications or death.\textsuperscript{4} The long-term incubation period of food poisoning by EHEC follows a typical pattern. The incubation period for diarrheal illness is usually 1 to 6 days, with a reported range of 1 to 14 days.\textsuperscript{5}

Recently, the mass outbreak of food poisoning caused by EHEC was one of the severe public health problems in Japan. Therefore, it would be very important to take countermeasures against outbreak of EHEC. As the countermeasure to the food-poisoning, there are known some prevention method.\textsuperscript{6} One of the most effective prevention methods must be enough heating of foods in cooking or preservation of the quality or freshness of food products. To prevent food-associated outbreaks, we have assessed the effect of chemical preservatives on the growth of EHEC.\textsuperscript{7} In summer season in which may increase the number of food-poisoning patients, the inhibition of the bacterial growth or production of enterotoxin such as verotoxin by any suitable methods would be also very important for the prevention countermeasure of food poisoning. The administration of some antibiotics would be also one of a suitable method for a cure of the infection by EHEC. Pros and cons on the administration of antibiotics have still been discussed and there would be some confusion in the medical field.

Before EHEC produces verotoxin in the human intestine, an agent inhibiting production of verotoxin should be administered to prevent food poisoning as one of effective prevention countermeasures.
In the previous paper,\(^7\) we examined the inhibitory effect of the creosote, a mixture of phenolic compounds, on the production of verotoxin (VT1 and VT2) by EHEC, and found that it inhibited verotoxin production. Furthermore, we also reported the inhibitory effect of clove, a famous crude drug on verotoxin.\(^8\)

As our subsequent study, we investigated the inhibitory effect of extracts from some North and Central American plants on the reduction of verotoxin by EHEC.

### MATERIALS AND METHODS

#### Test Sample

Twenty-one of plant samples growing wild were collected by the authors in Oregon of North America and in Mexico, Guatemala, and Honduras of Central America in summer seasons of both 1997 and 1998. The voucher specimens of the plant samples are deposited in the Herbarium of the Botanical Gardens, Faculty of Science, University of Tokyo.

#### Preparation of the Test Samples

The air-dried plant samples were first divided into several parts; i.e., stems, leaves, fruits, underground part, etc. The samples of secretary species were soaked in acetone, and the plant residues were extracted twice with methanol (MeOH) and then 70% aqueous (= aq.) MeOH at room temperature for 2 weeks. The samples of non-secretary species were, without washing in acetone, extracted twice (or three times) with MeOH and 70% aq. MeOH at room temperature for 2 weeks. Evaporation of the solvents in vacuo gave acetone, MeOH, and 70% aq. MeOH extracts (total 60 extracts). Twenty-two extracts of the 60 extracts were used as the test samples for the present bioassay (Table 1).

The 22 extract samples were not selected at random from the 60 extracts, but were selected by considering the results in preliminary antibacterial tests of the 60 extracts against some other microorganisms, i.e., *Staphylococcus aureus* IID 1677(MRSA) etc.\(^9\)

#### Test Bacteria and Medium

*Escherichia coli* ATCC 43889 (EHEC, VT2-producing strain) was
Table 2. Effect of the Plant Extracts on Growth of Escherichia coli ATCC 43889

<table>
<thead>
<tr>
<th>Samples</th>
<th>Control</th>
<th>Concentration of plant extract (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>31.3</td>
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<tr>
<td>No.7</td>
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<td>1.09 × 10⁹</td>
</tr>
<tr>
<td>No.9</td>
<td>8.75 × 10⁸</td>
<td>1.08 × 10⁹</td>
</tr>
<tr>
<td>No.10</td>
<td>8.75 × 10⁸</td>
<td>1.01 × 10⁹</td>
</tr>
<tr>
<td>No.11</td>
<td>8.75 × 10⁸</td>
<td>1.91 × 10⁹</td>
</tr>
</tbody>
</table>

BNo: Bacterial numbers of EHEC in control (CFU/ml)
BNt: Bacterial numbers of EHEC in each of the test tubes (CFU/ml)

Measurement of the Amount of Verotoxin Produced by EHEC —— Ten µl of EHEC overnight culture medium (approximately 1.00 × 10⁸ CFU/ml) at 37°C was added to test tubes containing 4.5 ml of CAYE broth and 0.5 ml of each of the plant extracts (final concentrations of 31.2, 62.5, 125, 250 and 500 µg/ml). Then the test tubes were incubated for 24 hr at 37°C with shaking (110–120 rpm). The cultures were centrifuged at 5°C (2100 × g for 15 min), and the amount of verotoxin in the supernatant fraction was measured by latex agglutination reaction using VTEC-RPLA “SEIKEN” (Denka Seiken Co. Ltd., Tokyo, Japan).¹¹)

The amount of verotoxin production in the presence of each plant extract was calculated from the following formula:

\[
\text{Amount of the production of verotoxin (ng/ml)} = \text{DDV} \times \frac{\text{BNo}}{\text{BNt}}
\]

DDV: Detection dose of verotoxin (ng/ml)
BNo: Bacterial numbers of EHEC in control (CFU/ml)

RESULTS AND DISCUSSION

Effect of the Plant Extracts on the Growth of EHEC

To make sure the effect of the plant extracts on the growth of EHEC, the MIC values of the extracts were determined by the agar dilution method. The MICs of the samples were same as 1000 µg/ml (final concentration), which indicating that plant extracts showed the antibacterial activity (data not shown).

When EHEC (approximately 8.75 × 10⁸ CFU/ml) was incubated with the plant extracts at the concentrations less than MIC, no decrease of bacterial numbers was observed as shown in Table 2. Therefore, we carried out the following experiments using less then 500 µg/ml of the plant extracts.

The Inhibitory Effects of the Plant Extracts on the Production of Verotoxin of EHEC

We then examined the inhibitory effect of the plant extracts on the production of verotoxin (VT2) by EHEC (Table 3). When the amount of the production of verotoxin in the test group was 4 times smaller than that of control, we judged the inhibitory effect of each of plant extracts as effective.

Among the plant extracts tested, samples of No. 7, 9, 10 and 11 prevented the production of verotoxin, and their minimal inhibition doses of verotoxin production were 125, 62.5, 125 and 31.3 µg/ml, respectively. Sample No. 11 showed the most potent inhibition for verotoxin production.

The dose required for the inhibition of verotoxin production was remarkably lower than that of MIC, and no decrease of bacterial numbers was observed.
Verotoxins or Shiga-like toxins are holotoxins composed of a single enzymatic A subunit of approximately 32 kDa in association with a pentamer of receptor-binding B subunit of 7.7 kDa. The expression of the A- and B-subunit genes is differently regulated: production of verotoxins is negatively regulated at the transcriptional level by an iron-Fur protein corepressor complex. Many factors such as growth conditions including composition of medium, antibiotics, and aeration are affected on the production level of the toxins or release of the toxins into the outside of the cells.

In our present study, it is deduced that 4 kinds of test plant extracts would inhibit the production process of verotoxin of EHEC specifically without any effects on the cell growth. The fine mechanism of the production of the verotoxins has been unresolved so far; plant extract would act directly or indirectly interfere the transcriptional and/or translational steps and reduce the production of the toxins. The findings described here suggest that administration of the plant extracts will prevent the production of verotoxin on EHEC in the human intestines.

The four plant species, the extracts of which gave the inhibitory effect, are all endemic to the Central America. They are also known as folk medicines and are as follows: Limonium californicum (Boiss.) A. Heller has a narrow distribution range along the coast of California, U.S.A., and has been used by Native American people as blood medicine, a remedy for urinary and venereal disease, etc. Some other Limonium species are also used as popular folk medicines in China and Taiwan. Cupressus lustianica Miller is distributed in Central America from Mexico to Guatemala. This woody plant is very prized as timbers and also for making musical instrument so that it is widely planted. Essential oils are obtained from some species in this genus. Salvia urica Epling is distributed in Mexico, Guatemala and Honduras. Many species in genus Salvia are used as foods and medicines. Jusiaea peruviana L. is distributed widely in Central America and adjacent South America and Florida. It is locally used as foods and folk medicines.

From the plant extracts giving the inhibition of verotoxin production on EHEC, the investigation and isolation of the active components should be undertaken as our subsequent study in the future. Attempts to clarify the mechanism responsible for the inhibitory effect of verotoxin production of EHEC by these plant extracts are now under study.

### REFERENCES


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<th>Samples</th>
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</tr>
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