Effects of *Psidium guajava* leaf extract on secretion systems of Gram-negative enteropathogenic bacteria

Running title: Guava extract and secretion system

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ABSTRACT

We screened a total of 672 plant-tissue extracts to search for phytochemicals that inhibit the function of the type III secretion system (T3SS) of enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). Among candidates examined, we found that an extract from the leaves of *Psidium guajava* (guava) inhibited the secretion of the EspB protein from EPEC and EHEC without affecting bacterial growth. The guava extract (GE) also inhibited EPEC and EHEC from adhering to and injecting EspB protein into HEp-2 cells. GE seemed to block the translocation of EspB from the bacterial cells to the culture medium. In addition to EPEC and EHEC, GE also inhibited the T3SS of *Yersinia pseudotuberculosis* and *Salmonella enterica* serovar Typhimurium. After exposure to GE, *Y. pseudotuberculosis* stopped the secretion of Yop proteins and lost its ability to induce the apoptosis of mouse bone marrow-derived macrophages. *S.* Typhimurium exposed to GE ceased the secretion of Sip proteins and lost its ability to invade HEp-2 cells. GE inhibited EspC secretion, the type V secretion protein of EPEC, but not Shiga toxin2 from

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EHEC. Thus, our results suggest that guava leaves contain a novel type of antimicrobial compound that could be used for the therapeutic treatment and prevention of gram-negative enteropathogenic bacterial infections.

Keywords leaf extract, *Psidium guajava*, type III secretion system

Enteropathogenic *Escherichia coli* (EPEC) is one of the major causes of infant diarrhea in developing countries. All the genes required for the formation of attaching and effacing (AE) lesions are encoded by the locus for the enterocyte effacement (LEE) pathogenicity island, which also encodes the bacterial type III secretion system (T3SS) (**1**). This system allows bacteria to deliver effector proteins into eukaryotic host cells. The EspA, EspB, and EspD proteins construct the T3SS apparatus to translocate the translocated intimin receptor (Tir) protein, which is a receptor for the outer membrane protein intimin (2). As EspB is the most abundant T3SS protein, a reversed passive latex agglutination test (3) and a rapid immunochromatographic test (4) detecting EspB are utilized as diagnostic tools for EPEC infection.

Enterohemorrhagic *E. coli* (EHEC) is a zoonotic pathogen, which causes a wide spectrum of illnesses ranging from mild diarrhea to severe diseases such as hemorrhagic colitis and hemolytic-uremic syndrome (HUS). HUS is the leading cause of acute pediatric renal failure and is associated with Shiga toxins (5). EHEC also has an LEE gene and secretes proteins to induce AE lesions similar to those induced by EPEC. T3SS is a major virulence factor of EPEC

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and EHEC as well as other Gram-negative pathogens, such as *Salmonella*, *Shigella, Yersinia*, and *Bordetella* (6,7).

Small-molecule inhibitory factors that bind and inhibit the T3SS have attracted the attention of researchers (8 -12). Plant products are currently considered to be important alternative sources of new antimicrobial drugs to fight microorganisms that have acquired resistance to classical antibiotics (13,14). Here, we describe a plant extract that inhibits the T3SS of EPEC and EHEC as well as that of *Y. pseudotuberculosis* and *S. enterica* serovar Typhimurium.

MATERIALS AND METHODS

Plant extracts

A total of 672 plant-tissue extracts were kindly provided by The Okinawa Industrial Technology Center. To prepare these extracts, 4 g of freeze-dried plant tissue was soaked in 20 mL of 50% ethanol and heated at 80°C for 1 h, then filtered with filter paper.

Antibody and recombinant protein

The anti-EspB antiserum was prepared as described by Lu *et al.* (3). Antibodies against SipB and SipC were provided as described by Gebauer *et al*. (15). Anti-caspase-3 antibody was obtained commercially (#9662S Cell Signaling). Recombinant EspB protein was prepared by Lu *et al*. (3). Anti-EspC or anti-Shiga toxin 2 antibody was prepared as described by Drago-Serrano *et al.* (16) and Yutsudo *et al.* (17), respectively.

Bacterial strains

The bacteria used in this study were EPEC strain E2348/69 (O127:H6), EHEC

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strain EDL933 (O157:H7), *Y. pseudotuberculosis* virulent strain (18), and *S.* Typhimurium virulent strain (19). EPEC *escN* deletion mutant, a T3SS-deficient mutant, was provided by Professor Abe of Kitasato University. To induce the T3SS, EPEC and EHEC were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C for 5 hr in 5% CO_2, *Y. pseudotuberculosis* was grown in Luria-Bertani (LB) broth supplemented with 20 mM sodium oxalate and 20 mM $MgCl_2$ at 37°C for 4 hr (20), and *S*. Typhimurium was grown in LB supplemented with 0.3 M NaCl at 37°C for 3 hr (21).

Initial screening and assays to detect EspB

For the initial screening, EPEC or EHEC was grown in DMEM with each plant extract at a final concentration of 0.25% at 37°C for 5 hr, and the bacterial cells were then removed by centrifugation at 20,000 *g* for 10 min. At the same time, we prepared two types of controls: a culture supernatant of EPEC or EHEC grown in the same medium without any plant extract under the same conditions, and a culture supernatant from *escN*-mutant EPEC grown in the same medium without any plant extract under the same conditions. The amount of EspB in each culture supernatant was quantified using an enzyme-linked immunosorbent assay (ELISA) according to the method described by Clark and Adams (22). Briefly, each well of the ELISA plates was coated with each culture supernatant, and EspB was detected using anti-EspB antiserum, horseradish peroxidase labeled anti-rabbit IgG, and the coloring reagent 5-aminoacetoacetic acid, which can be measured based on the optical density at 450 nm (OD450). To evaluate the suppression of EspB by a plant extract, an index, EspB%, was calculated using the following formula: EspB% = {(B-C)/(A-C)} x100, in which A

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is the OD450 value obtained by culturing without the plant extract, B is the value obtained by culturing with the plant extract, and C is the one obtained from the *escN* mutant. To detect EspB in the culture supernatant, the total protein was precipitated with 10% (final concentration) trichloroacetic acid (TCA) at 4°C for 1 hr, and the precipitates were separated using sodium dodecyl sulfate 15%-polyacrylamide gel electrophoresis (SDS-PAGE) and examined using Western blotting with anti-EspB antiserum; alternatively, protein staining was performed using Coomassie Brilliant Blue. The EPEC *escN* deletion mutant was used as a negative control, and bacteria cultured in DMEM without plant extracts were used as a positive control to confirm the inhibitory effect of plant extracts on EspB expression.

Protease activity assay

Purified recombinant EspB (5 mg/mL) was mixed with 0.25% guava leaf extract (GE) and incubated in 10 mM Tris-HCl (pH7.5) at 37°C for 1- 8 hr. Trypsin (100 μg /mL) was used as a positive control enzyme. Protease activity of GE on casein or gelatin was detected by using a single-diffusion technique in agar gel containing 1% skim milk as a substrate (23) or 0.01% gelatin zymography (24). For the single-diffusion assay, GE or trypsin (10 μl respectively) was added to wells 3 mm in diameter, and the plates were incubated at 37°C for 20 hr. The gelatin zymography was performed as described previously (24).

Reverse transcription-polymerase chain reaction (RT-PCR) for *espB* and *escN* genes

To evaluate the transcription of the *espB* gene in EPEC or EHEC, RT-PCR was

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performed as previously described (25). Briefly, the total RNA was prepared from bacteria cultured in DMEM supplemented with or without 0.25% GE for 5 hr at 37°C (OD600 nm = 0.85 ± 0.05, late log phase) using an RNA isolation kit (RNeasy Mini kit; Qiagen, Valencia, CA), and the complementary DNA was prepared with an RT-PCR kit (SuperScript III One-Step Reverse Trans-PCR System; Invitrogen, CA). Subsequent PCR for *espB* amplification was performed using a set of primers of B148 (5'-GCCGTTTTTGAGAGCCA-3') and B151 (5'-TCCCCAGGACAGATGAGAT-3') for the type-α *espB* of EPEC; and of B148 and B150 (5'-GCACCAGCAGCCTTTGA-3') for the type-γ *espB* of EHEC (26) under condition with 25 cycles of 94°C for 20 s, 55°C for 40 s, and 72°C for 2 min. To examine the expression of *escN*, a gene encodi-3'ng a protein secreted by the T3SS, PCR with a set of primers of N1 (5'-CGCCTTTACAAGATAGAAC-3') and N2 (5'-CATCAAGAATAGAGCGGAC-3') was performed under conditions with 35 cycles of 93°C for 1 min, 54°C for 40 s, and 72°C for 1 min (27). An RNA sample without reverse transcriptase was included in a series of RT-PCR, to confirm that the corresponding DNA contaminants were not contained in an RNA sample. The PCR products were analyzed by electrophoresis using 2% agarose gel.

Evaluation of the cell adhesion activity of EPEC and EHEC and detection of EspB in the host cells

To test the cell adhesion activity of EPEC or EHEC, HEp-2 cells (2×10^5 cells/well) were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 for 18 hr, and EPEC or EHEC were cultured in DMEM supplemented with or without 0.25% GE at 37°C in 5% CO_2 for 2 hr. The

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bacteria were spun down and resuspended in DMEM, and then added to HEp-2 cells at a multiplicity of infection (MOI) of 100. After 2 hr of co-culture at 37°C in 5% CO_2, the HEp-2 cells were washed with PBS, fixed with methanol, and stained with Giemsa. The number of adhered bacteria was counted for approximately 100 HEp-2 cells in 10 randomly selected microscopic fields.

To examine the translocation of EspB into the host cells, HEp-2 cells were infected with EPEC or EHEC for 2 hr as described above for the cell adhesion assay, and the plasma membrane of the HEp-2 cells was then isolated using the methods reported by Dignam *et al.* (28). Briefly, HEp-2 cells infected with bacteria were washed with 10 mM HEPES-NaOH (pH7.9), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT. The cells were then collected with a scraper. After disrupting the collected cells with a homogenizer, the disrupted cells were centrifuged at 100,000 *g* at 4°C for 1 hr to obtain the supernatant (cytoplasmic fraction) and pellet. The pellet was solubilized with 1% Tween 20 and used as a plasma membrane fraction. The cytoplasmic and plasma membrane fractions were analyzed using Western blotting as described above.

Subcellular fractionation of bacterial cells

The subcellular fractionation of the bacterial cells was performed as previously described (29). Briefly, bacteria cultured in DMEM supplemented with or without GE were harvested by centrifugation at 10,000 *g* for 10 min. The bacterial pellets were suspended in 20% sucrose and 50 mM Tris-HCl (pH7.0). Cells were treated with 10 mM EDTA and 100 μg/mL of lysozyme for 10 min at room temperature. The periplasmic fraction was obtained from the supernatant obtained by centrifuging the cell suspension at 8,000 *g* for 10 min. The pellet

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was resuspended in 10 mM Tris-HCl (pH7.0) and sonicated three times for 15 sec. Unbroken bacteria were removed by centrifugation at 16,000 *g* for 2 min, and the supernatant was centrifuged for 1 hr at 50,000 *g*. The supernatant was pooled as the cytoplasmic fraction. The pellet was resuspended in 0.5% *N*-lauroylsarcosine to solubilize the inner membrane and centrifuged at 50,000 *g* for 1 hr. The supernatant and the pellet were supposed to contain the inner membrane protein and the outer membrane, respectively. All the samples were suspended in SDS-PAGE sample buffer supplemented with β-mercaptoethanol and heated at 100°C for 5 min to detect EspB using Western blotting.

Analysis of T3SS of *Y. pseudotuberculosis* or *S.* Typhimurium

To examine the T3SS-related proteins secreted into the culture supernatant, the supernatant was collected by spinning down the bacterial cells at 20,000 *g* for 10 min. To identify the Yop proteins in the culture supernatant from *Y. pseudotuberculosis*, the total protein was concentrated by TCA precipitation, separated by SDS-PAGE, and visualized using Coomassie Brilliant Blue staining as reported previously (30, 31). Two kinds of T3SS proteins from *S.* Typhimurium, SipB and SipC, were identified using Western blotting with specific antibodies against these proteins.

Detection of apoptosis induced by *Y. pseudotuberculosis*

To evaluate the ability of *Y. pseudotuberculosis* to induce apoptosis in eukaryotic host cells, bone marrow-derived macrophages (BMMs) were prepared from C57BL/6 mice, and *Y. pseudotuberculosis* was cultured in T3SS-inducing medium supplemented with or without GE for 4 hr. Bacteria collected by centrifugation were resuspended with DMEM and then added to the BMMs

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at MOI of 50:1. After 2 hr of co-culture, the apoptotic cell death of the host cells was analyzed using the DeadEnd Fluorometric TUNEL (terminal deoxynucleotidyl transferase–mediated nick-end labeling) system (Promega Corp. Wisconsin, USA), as recommended by the manufacturer.

To detect the activation of caspase-3, which is associated with apoptosis, BMMs were infected with *Y. pseudotuberculosis* as described above. Cells were lysed by adding 1% Tween 20 to the culture medium, and the total protein including the protein secreted from the BMMs was precipitated from the lysate using 10% TCA. The precipitates were re-solubilized in the SDS-PAGE loading buffer and applied to a 15% SDS-PAGE gel. The cleaved form of caspase-3 was detected using anti-caspase-3 antibody. A cell lysate prepared from BMMs exposed to staurosporine (1 μM), a reagent that induces apoptosis, was used as a positive control for the activated caspase-3.

Gentamycin protection assay

The gentamycin protection assay was used to detect the intracellular infection of *S.* Typhimurium, as previously described (32).

Statistical analysis

All the experiments were performed two or three times in triplicate. Data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using unpaired two-tailed Student *t*-tests. Differences were considered significant at a P value of <0.05.

RESULTS

Screening for plant-tissue extracts that inhibit EspB secretion

Since the ethanol used in the preparation of the plant extracts may affect

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bacterial growth, we first examined the effect of ethanol at different concentrations (1, 0.5, 0.25, and 0.125%) on bacterial growth and EspB secretion. The growth of neither EPEC nor EHEC was inhibited at a 1% ethanol concentration, and the EspB secretion of neither pathogen was affected by ethanol concentrations of up to 0.25%. Based on these results, we decided to use plant extracts at a final concentration of 0.25% to screen for T3SS inhibitors. An initial screening of 672 samples revealed that an extract from the leaves of *Psidium guajava* (guava) (abbreviated as GE) inhibited the secretion of EspB from both pathogens without obvious growth inhibition. The inhibition ratio of EspB secretion, EspB%, for GE ranged from 82 to 100 (data not shown). We confirmed the inhibitory effect of GE on the secretion of EspB using SDS-PAGE (Fig. 1a). When bacteria were cultured in a medium without GE, EspC (serine protease) and EspA (needle component of T3SS) proteins were observed in the culture supernatant in addition to EspB. However, no proteins were observed in the culture supernatant of bacteria cultured in a medium containing 0.5% or 0.25% GE, indicating that GE inhibits T3SS proteins secretion (Fig. 1a). The inhibition of EspB secretion by GE was also examined using ELISA (Fig. 1b). The results showed that the inhibitory effect of GE on EspB secretion was dose-dependent (Fig. 1a, b). We confirmed that GE did not affect the growth of either EPEC or EHEC for up to 8 hr (Fig. 1c). Since we considered that GE might contain a protease activity, we incubated recombinant EspB protein with 0.25% GE for 1 to 8 hr. The results of Western blotting (Fig. 1d) showed that the EspB in the culture supernatant of EPEC was not degraded by the exposure to GE. The proteolytic ability of GE on casein and gelatin were

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examined by using single diffusion assay or gelatin zymography. As a result, we could not detect protease activity in the GE preparation (Fig.1e, 1f).

Transcription of the *espB* and *escN* gene expression, and subcellular localization of the EspB protein

Bacterial RNA was harvested in the same amount with a concentration approximately at 30 µg/mL regardless of the presence of 0.25% GE. Transcriptional levels of *espB* and *escN* genes were examined by RT-PCR using the extracted RNA in the presence or absence of GE. Amplicons corresponding to *espB* subtype α (188 bp) for EPEC or subtype γ (233 bp) for EHEC, and to *escN* (856 bp) for EPEC were produced to the same extent with or without GE (Fig. 2a, 2b), indicating that the transcription activity of either *espB* or *escN* were not influenced by the presence of 0.25% GE. Corresponding amplicons were not produced from the RNA samples lacking reverse transcriptase, confirming that DNA fragments containing these two genes were not contaminated in the samples (data not shown). To investigate the secretion pathway through T3SS of the bacteria cultured with or without GE, bacteria were fractionated into outer-membrane (OM), inner-membrane (IM), periplasmic (P), and cytoplasmic fractions (Cy), and the localization of the EspB protein was examined using Western blotting. As shown in Fig. 2c, EspB was detected in the inner membrane and cytoplasmic fractions regardless of the presence or absence of GE.

Effect on bacterial adhesion and EspB translocation

If the T3SS does not work, bacterial adhesion to cells might be reduced, since the T3SS is involved in cell adhesion. In addition, if EspB cannot be

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translocated into HEp-2 cells, EspB would not be found within those cells. To examine these matters, the adhesion of the bacteria to HEp-2 cells and EspB localization within the cells were examined. The adhesion of both pathogens to HEp-2 cells was inhibited to within a range of approximately 80% to 90% in the presence of GE (Fig. 3a). HEp-2 cells were infected with EPEC cultured with or without GE, and the intracellular distribution of EspB was examined. When the HEp-2 cells were examined using Western blotting, EspB was found in the plasma membrane (PM) and cytoplasmic (Cy) fractions only in the cells infected with the bacteria cultured without GE (Fig. 3b).

Effects on other EPEC and EHEC strains

Using GE, other EPEC and EHEC strain stored in our laboratory (Table 1) were tested to observe whether EspB secretion was inhibited. The results showed that EspB secretion from the tested bacteria was inhibited by GE (Fig. 4a, 4b).

Effects on other bacterial T3SS

To test the effect of GE against other bacterial T3SS, *Y. pseudotuberculosis* and *S.* Typhimurium were examined. The secretion of the translocator proteins of *Y. pseudotuberculosis*, YopH, YopB, and YopD, was inhibited by GE without inhibiting bacterial growth (Fig. 5a, 5b). *Y. pseudotuberculosis* is known to induce apoptosis in macrophages via T3SS (20). We examined whether GE inhibited such apoptosis. Since the caspase cascade is induced by the apoptotic process, we examined caspase-3 activation using Western blotting. The macrophages treated with staurosporine, a reagent used to induce apoptosis, and infected with *Y. pseudotuberculosis* showed caspase-3 activation, but the macrophages infected with *Yersinia* cultured with GE did not

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(Fig. 5c). We also confirmed apoptosis using the TUNEL reaction to detect the presence of DNA fragmentation (Fig. 5d). The TUNEL-positive percent rates were 2.6 ± 5.1, 80 ± 4.4, and 2.3 ± 0.6 (mean ± SD) for no infection, infection by bacteria cultured in LB broth supplemented without GE, and infection by bacteria cultured in LB broth supplemented with GE, respectively (Fig. 5e). In the case of *S*. Typhimurium, the secretion of the T3SS translocator proteins SipB and SipC was inhibited by GE without inhibiting bacterial growth (Fig. 6a, 6b). *Salmonella* pathogenicity island-1 (SPI-1) encodes T3SS and is essential for cell invasion. T3SS inhibition by GE might reduce bacterial cell invasion. We examined the invasion of HEp-2 cells by *Salmonella* using a gentamycin protection assay. The invasiveness of *Salmonella* cultured with GE was reduced, ranging from 0% to 3% (Fig. 6c).

Effects of GE on other type secretion systems

In Fig.1a, a band estimated to be the EspC protein, an autotransporter protein secreted via the type V secretion system (T5SS), also reduced its intensity in response to GE. We, therefore, conducted a Western blotting to confirm the kinetics of EspC. As a result, secretion of EspC was also inhibited by GE in a dose-dependent manner (Fig. 7a), suggesting that the GE inhibits not only T3SS but also T5SS.

Since EHEC is known to secrete Shiga toxins (Stx1 and Stx2), which are pathogenic factors for dysentery, we have also investigated the effect of GE on the secretion of Stx2 by Western Blotting. As shown in Fig. 7b, GE did not affect the secretion of Stx2 from EHEC, which suggested that the GE affect only certain kinds of secretion systems but not all.

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DISCUSSION

Diarrheagenic *E. coli*, including EPEC and EHEC, are common pathogens that cause diarrhea in children in developing countries (33). Both pathogens are reported to use T3SS in intimate attachment to intestinal epithelial cells, and to inject effector proteins into the host cells to cause disease (34, 35). Besides the diarrheagenic *E. coli*, many other enteropathogenic bacteria that belong to Family *Enterobacteriaceae*, such as *Y. pseudotuberculosis* and *S*. Typhimurium, possess the T3SS as a major virulent factor. We believe that a compound able to suppress a T3SS function might be a promising option to expect a synergic effect with precedent therapeutic compounds including antibiotics in cases of infectious diseases caused by T3SS-carrying pathogens.

For this reason, we have screened 672 plant extracts to search for a novel antimicrobial compound supposed to inhibit T3SS from secreting EspB proteins of EPEC and EHEC, and finally found that an extract from *P. guajava* leaves revealed a capacity what we had expected (Fig. 1). In addition, a similar inhibition effect was observed on the T3SS carried by *Y. pseudotuberculosis* (Fig. 5a, 5b) or *S.* Typhimurium (Fig. 6a, 6b). Although leaves of *P. guajava* has been historically used as a folk medicine in Vietnam (36) and has been studied for its potential biological properties, this is the first report to describe that GE seems to have a property to inhibit the secretion of EspB proteins from T3SS carried by a wide variety of enteropathogenic bacteria across family *Enterobacteriaceae*.

Next, we attempted to elucidate a mechanism by which GE inhibits the T3SS.

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Because Ochoa *et al.* previously reported that lactoferrin in mammalian milk blocked T3SS-mediated actin polymerization and hemolysin by EPEC, degrading the secreted proteins (37), we examined whether GE possesses protease activity by incubating recombinant EspB protein with GE. As shown in Fig. 1d, the EspB protein was not digested by GE, and GE did not digest casein or gelatin (Fig. 1e and 1f), suggesting that GE inhibits the T3SS in a manner different from that of lactoferrin. To investigate the effect of GE on the secretion machinery through the T3SS, we fractionated the bacterial cells into outer-membrane, inner-membrane, periplasmic, and cytoplasmic fractions and examined the subcellular localization of the EspB protein using Western blotting. As shown in Fig. 2, EspB was detected in the inner membrane and cytoplasmic fractions regardless of whether GE was present or not. These results suggest that the GE does not affect the translation and accumulation of EspB protein in the bacterial cells, but rather blocks the translocation of the EspB protein from the bacterial cytoplasm into the extracellular space. We also investigated whether GE inhibits the bacterial pathogenicity known to be associated with the T3SS using EPEC, EHEC, *Y. pseudotuberculosis* and *S.* Typhimurium. As shown in Fig. 3a, GE inhibited the adhesion of EPEC and EHEC to the mammalian host cells, which was thought to occur through the blockade of T3SS. As shown in Fig. 5d and 5e, GE inhibited apoptotic cell death induced by the T3SS of *Y. pseudotuberculosis*. As shown in Fig. 6c, GE also inhibited the intracellular infection of *S.* Typhimurium.

GE was shown to inhibit not only T3SS but also the T5SS (Fig. 7a). However, GE did not inhibit secretion of Stx2 (Fig. 7b). These results suggested that GE

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inhibits a common mechanism between the T3SS and T5SS, which does not exist in the secretion machinery of Stx.

In conclusion, GE apparently contains a novel, wide-range spectrum inhibitor of the T3SS. Although the mechanism requires further elucidation, the active component of guava extract might be a good candidate for a therapeutic drug for infectious diseases caused by T3SS-positive enteropathogenic bacteria.

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DISCLOSURES

No conflicts of interests are declared.

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FIGURE LEGEND

Fig.1. Effects of *P. guajava* leaf extracts on T3SS. (a) SDS-PAGE protein staining and (b) ELISA assay of EspB of EPEC or EHEC in the culture supernatant. Bacteria were cultured in DMEM with or without (-) *P. guajava* leaf extracts (GE). The concentration of GE (% GE) was reduced from 0.5 % to 0.06 %, and EspB secretion inhibition was examined. The bar graphs represent the mean ELISA assay and show EspB %. Error bars, SD of triplicate samples. ∗; $p < 0.05$ versus control. (c) The effect of 0.25% GE on bacterial growth in the medium supplemented with (●) or without (○) GE. Bacterial growth was monitored every 2 hr spectrophotometrically at the OD 600 nm. (d) Protease activity of 0.25% GE on EspB. Recombinant EspB has treated with 0.25% GE for 1 to 8 hr and analyzed by Western blotting. Trypsin (Tryp, 100 μl/ml) was used as a positive control and nontreatment (-) was used as a negative control. (e) Detection of protease activity by skim milk agar. Ten μl of GE or Trypsin (Tryp) was added to wells and incubated at 37°C for 20 hr. The clear zone indicates that casein has been digested. (f) Detection of protease activity on gelatin zymography. After electrophoresis of GE and trypsin (Tryp), the gel was incubated at 37°C for 20 hr. The clear band indicates that gelatin has been digested.

Fig. 2. Transcription of the *espB* gene and localization of the EspB protein. The reverse transcription-polymerase chain reaction (RT-PCR) of *espB* (a) or

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escN (b) mRNA in the bacteria grown with (+) or without (-) 0.25% GE. *espB* α (EPEC; 188 bp), *espB* γ (EHEC; 233 bp) and *escN* (856 bp) in bacterial cytosol were detected by RT-PCR. (c) EspB localization in the bacteria grown with (+) or without (-) 0.25% GE. Bacteria cultured with or without GE were fractionated into culture supernatant (Sup), outer membrane (OM), periplasmic (P), inner membrane (IM) and cytoplasmic (Cy) fractions, and EspB was detected by Western blotting. *ΔescN* (-); T3SS mutant strain cultivated without GE.

Fig. 3. Effect of 0.25% GE on bacterial adhesion and EspB injection. (a) Relative adhesion (percent ratio, related to the untreated control) of EPEC or EHEC to HEp-2 cells. Bacteria were cultured in DMEM supplemented with (GE) or without (-) GE. EPEC *ΔescN* (T3SS mutant) strain was used as the T3SS negative control. HEp-2 cells were infected with the bacteria. Adherent bacteria were counted by microscopic observation. Values are mean ± SD of triplicate samples. *; $p < 0.05$ versus control. (b) Western blotting of EspB in HEp-2 cells. After HEp-2 cells were infected with bacteria cultured supplemented with (+) or without (-) GE, the cell was fractionated into the plasma membrane (PM) and cytoplasmic (Cy) fractions. EspB was detected by Western blotting. NI; Non-infection HEp-2 cell fractions.

Fig. 4. The effect of 0.25% GE on other EPEC and EHEC strains. (a) Western blotting and (b) ELISA assay of the effect of GE on other EPEC and EHEC strains. The different EPEC or EHEC strains listed in table 1 were cultivated in the medium with (+) or without (-) GE. After cultivation, EspB in culture supernatants was examined as described in the materials and methods. *; $p < 0.05$ versus without extracts.

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Fig. 5. The effect of 0.25% GE on *Y. pseudotuberculosis* T3SS. (a) The growth of *Y. pseudotuberculosis* cultured in LB medium supplemented with (●) or without (○) GE. Bacterial growth was monitored every 2 hr spectrophotometrically at the OD 600 nm. (b) Protein staining of *Y. pseudotuberculosis* T3SS proteins. *Y. pseudotuberculosis* was grown in LB broth supplemented with (+) or without (-) GE. The T3SS proteins, YopH, YopB, and YopD, in the culture supernatant, were examined. Each protein was estimated by molecular weight. MM; molecular weight marker. (c) The effect of the extracts on caspase-3 activity in macrophages. Macrophages were infected with *Y. pseudotuberculosis* for 2 hr and the active caspase-3 was detected by Western blotting. NI; Non-infection, Sta; staurosporine (1 μM), *Yersinia* cultured in LB broth supplemented with (+) or without (-) GE infection. The staurosporine-treated macrophage was used as an apoptosis positive control. (d) Induction of apoptosis in the *Yersinia* infective-mouse bone marrow-derived macrophages (BMMs) as determined by the TUNEL assay. TO-PRO3; a nucleic acid-binding dye that stains early apoptotic and necrotic cells differentially. TUNEL; terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. Sta; staurosporine (1μM). *Yersinia* GE (-); *Y. pseudotuberculosis* cultured without GE. *Yersinia* GE (+); *Y. pseudotuberculosis* cultured with GE. Scale bar, 10 μm. (e) The percent ratio of TUNEL-positive cells to total cell number. The number of cells was obtained from the TUNEL image (Fig 5d). NI; Non-infection. GE (-); Infection by bacteria cultured without GE, GE (+); Infection by bacteria cultured with GE. ∗; $p < 0.05$ versus without extracts.

Fig. 6. Effect of 0.25% GE on *S.* Typhimurium T3SS. (a) The growth of *S.*

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Typhimurium cultured in LB medium supplemented with (●) or without (○) GE. Bacterial growth was monitored every 2 hr spectrophotometrically at the OD 600 nm. (b) Western blotting of SipB and SipC. *Salmonella* cultured in LB broth supplemented with (+) or without (-) GE. (c) Gentamycin protection assay. Macrophages were infected with *S.* Typhimurium cultured in a medium with GE (+) or without (-) GE. After killing extracellular bacteria with gentamycin, the intracellular bacteria in HEp-2 cells were cultured on an agar plate. The values were given as a percentage of the number of bacterial colonies grown without (-) GE. ∗; $p < 0.05$ versus without extracts.

Fig. 7. Effects on other type secretion systems. (a) Effects on the EspC secretion. The culture supernatant of EPEC cultured in DMEM containing 0.5 to 0.06% GE was examined by Western blot using the anti-EspC antibody or anti-EspB antibody. (b) Effects on Shiga toxin secretion. The culture supernatant of EHEC cultured in DMEM containing 0.5 to 0.06% GE was examined by Western blot using anti-Stx2 antibody.

List of abbreviation:

AE, attaching effacing; BMMs, mouse bone marrow-derived macrophages; Casp.3, caspase-3; EspB, EPEC secreted protein B; EHEC, Enterohemorrhagic *Escherichia coli*; EPEC, Enteropathogenic *Escherichia coli*; GE, Guava leaf extracts; HUS, hemolytic-uremic syndrome; Sip, *Salmonella* invasion protein; SPI-1, *Salmonella* Pathogenicity Island-1; Sta, staurosporine; Stx, Shiga toxin; T3SS, Type III secretion system; T5SS, Type V secretion system; TUNEL, TdT-mediated dUTP nick end labeling; Yop, *Yersinia* outer proteins.

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Table 1

Characteristics of pathogenic *E. coli* strains

	No.	Year	Location	O-type	gene		
					easA	*stx*	other†
EPEC	1	1981	Kenya	O119	+	-	-
	2	1999	Laos	O142	+	-	-
	3	2000	Indonesia	O119	+	-	-
	4	2000	Indonesia	O119	+	-	-
	5	2003	Japan	O128	+	-	-
	6	2003	Japan	O26	+	-	-
	7	2003	Japan	O25	+	-	-
EHEC	8	2003	Japan	O111	-	+	-
	9	2003	Japan	O111	-	+	-
	10	2003	Japan	O26	-	+	-
	11	2003	Japan	O26	-	+	-
	12	2003	Japan	O26	-	+	-
	13	2003	Japan	O26	-	+	-

The strains stored in our laboratory were isolated from various locations in various years. PCR target genes: *easA* for EspA, *stx* for Shiga-like toxin.
† Other genes; *aggR* for enteroaggregative *E.coli, est* and *elt* for enterotoxigenic *E.coli, ipaH* for enteroinvasive *E.coli* (35). (+) present, (-) absent gene

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