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ORIGINAL ARTICLE

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

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ABSTRACT

In this study, 672 plant-tissue extracts were screened 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, an extract from the leaves of *Psidium guajava* (guava) was found to inhibit secretion of EPEC-secreted protein B (EspB) from EPEC and EHEC without affecting bacterial growth. Guava extract (GE) also inhibited EPEC and EHEC from adhering to, and injecting EspB into, HEp-2 cells. GE seemed to block translocation of EspB from the bacterial cells to the culture medium. In addition, GE also inhibited the T3SS of *Yersinia pseudotuberculosis* and *Salmonella enterica* serovar Typhimurium. After exposure to GE, *Y. pseudotuberculosis* stopped secreting *Yersinia* outer proteins and was unable to induce apoptosis of mouse bone marrow-derived macrophages. *S. typhimurium* exposed to GE stopped secreting Sip proteins and was unable to invade HEp-2 cells. GE inhibited secretion of EspC, the type V secretion protein of EPEC, but not secretion of Shiga toxin 2 from EHEC. Thus, our results suggest that guava leaves contain a novel type of antimicrobial compound that could be used to treat and prevent gram-negative enteropathogenic bacterial infections.

Key words leaf extract, *Psidium guajava*, type III secretion system.

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

Enterohemorrhagic *E. coli*, a zoonotic pathogen, causes a wide spectrum of illnesses ranging from mild diarrhea to severe diseases such as hemorrhagic colitis and hemolytic–uremic syndrome. Hemolytic–uremic syndrome, the leading cause of acute pediatric renal

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List of Abbreviations: BMM, mouse bone marrow-derived macrophage; DMEM, Dulbecco's modified Eagle's medium; EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; EspB, EPEC-secreted protein B; GE, guava leaf extract; Sip, *Salmonella* invasion protein; Stx, Shiga toxin; T3SS, Type III secretion system; T5SS, Type V secretion system; TCA, trichloroacetic acid; Yop, *Yersinia* outer proteins.

failure, is associated with Shiga toxins (5). EHEC also has an *LEE* gene and secretes proteins that induce attaching effacing lesions similar to those induced by EPEC. T3SS is a major virulence factor of EPEC and EHEC as well as of 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 combat 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

The Okinawa Industrial Technology Center (Okinawa, Japan) kindly provided 672 plant-tissue extracts. 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 hr, then filtered with filter paper.

Antibody and recombinant protein

Anti-EspB antiserum was prepared as described by Lu *et al.* (3). Antibodies against SipB and SipC were prepared as described by Gebauer *et al.* (15). Anti-caspase-3 antibody was obtained commercially (#9662S; Cell Signaling Technology, Danvers, MA, USA). Recombinant EspB protein was prepared according to the method of Lu *et al.* (3), anti-EspC antibody as described by Drago-Serrano *et al.* (16) and anti-Shiga toxin 2 as described by Yutsudo *et al.* (17).

Bacterial strains

The bacteria used in this study were EPEC strain E2348/69 (O127:H6), EHEC 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 (Kitasako, Japan). To induce the T3SS, EPEC and EHEC were cultured in DMEM at 37°C for 5 hr in 5% CO_2, *Y. pseudotuberculosis* was grown in 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, after which the bacterial cells were removed by centrifugation at 20,000 *g* for 10 min. At the same time, two types of control were prepared: 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 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, HRP-labeled anti-rabbit IgG and the coloring reagent 5-aminoacetoacetic acid, which can be measured based on the OD_{450}.

To evaluate suppression of EspB by the plant extracts, an index, EspB%, was calculated using the following formula: $\text{EspB\%} = \{(B-C)/(A-C)\} \times 100$, in which A is the OD_{450} obtained by culturing without the plant extract, B is the OD_{450} 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) TCA at 4°C for 1 hr, after which the precipitates were separated using SDS-PAGE and examined using western blotting with anti-EspB antiserum; alternatively, protein was stained with 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 determine the inhibitory effecst of plant extracts on EspB expression.

Protease activity assay

Purified recombinant EspB (5 mg/mL) was mixed with 0.25% GE and incubated in 10 mM Tris-HCl (pH 7.5) 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 (each 10 μL) was added to 3 mm diameter wells and the plates incubated at 37°C for 20 hr. Gelatin zymography was then performed as described previously (24).

RT-PCR for *espB* and *escN* genes

To evaluate transcription of the *espB* gene in EPEC or EHEC, RT-PCR was performed as previously described (25). Briefly, total RNA was prepared from bacteria cultured in DMEM supplemented with or without 0.25% GE for 5 hr at 37°C ($OD_{600} = 0.85 \pm 0.05$; late log phase) using an RNA isolation kit (RNeasy Mini

Kit; Qiagen, Valencia, CA, USA), whereas the complementary DNA was prepared with an RT-PCR kit (SuperScript III One-Step Reverse Trans-PCR System; Invitrogen, Carlsbad, CA, USA). Subsequently, 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′-GCACCAG-CAGCCTTTGA-3′) for the type-γ *espB* of EHEC (26) under the following conditions: 25 cycles of 94°C for 20 s, 55°C for 40 s, and 72°C for 2 min. To examine expression of *escN*, a gene encoding a protein secreted by the T3SS, PCR with a set of primers of N1 (5′-CGCCTTTACAA-GATAGAAC-3′) and N2 (5′-CATCAAGAATA-GAGCGGAC-3′) was performed under the following conditions: 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 there were no corresponding DNA contaminants in the RNA sample. The PCR products were analyzed by electrophoresis using 2% agarose gel.

Evaluation of cell adhesion activity of EPEC and EHEC and detection of EspB in 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% FBS at 37°C in 5% CO_2 for 18 hr, whereas EPEC and EHEC were cultured in DMEM supplemented with or without 0.25% GE at 37°C in 5% CO_2 for 2 hr. The bacteria were spun down and resuspended in DMEM, and then added to HEp-2 cells at a 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 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, after which the plasma membrane of the HEp-2 cells was 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 dithiothreitol. The cells were then collected with a scraper. After disrupting the collected cells with a homogenizer, they were centrifuged at 100,000 *g* at 4°C for 1 hr to obtain the supernatant (cytoplasmic fraction) and a pellet. The pellets were 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

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 then suspended in 20% sucrose and 50 mM Tris-HCl (pH 7.0). 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 8000 *g* for 10 min. The pellets were then resuspended in 10 mM Tris-HCl (pH7.0) and sonicated three times for 15 s. Unbroken bacteria were removed by centrifugation at 16,000 *g* for 2 min and the supernatant centrifuged for 1 hr at 50,000 *g*. The supernatants were then pooled as the cytoplasmic fraction. The pellets were resuspended in 0.5% *N*-lauroylsarcosine to solubilize the inner membrane and centrifuged at 50,000 *g* for 1 hr. The supernatant and pellet were considered to contain the inner membrane protein and the outer membrane, respectively. All 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 Yop proteins from *Y. pseudotuberculosis* in the culture supernatant, total protein was concentrated by TCA precipitation, separated by SDS-PAGE and visualized using Coomassie Brilliant Blue staining, as reported previously (30, 31). Two types 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, 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 at an MOI of 50:1. After 2 hr of co-culture, apoptotic death of the host cells was analyzed using a DeadEnd Fluorometric TUNEL system (Promega, Madison, WI, USA), as recommended by the manufacturer.

To detect 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, precipitated from the lysate using 10% TCA. The precipitates were re-solubilized in 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

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

Statistical analysis

All the experiments were performed two or three times in triplicate. Data are expressed as the mean ± SD. Statistical analyses were performed using unpaired two-tailed Student *t*-tests. Differences were considered significant when $P < 0.05$.

RESULTS

Screening for plant-tissue extracts that inhibit EspB secretion

Because the ethanol used in the preparation of the plant extracts may affect bacterial growth, the effect of ethanol on bacterial growth and EspB secretion was first examined at different concentrations (1%, 0.5%, 0.25% and 0.125%). Both growth of EPEC and EHEC was not inhibited by a 1% ethanol concentration and ethanol concentrations of up to 0.25% did not affect EspB secretion by either pathogen. On the basis of these results, plant extracts were used 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) inhibited secretion of EspB by both pathogens without obvious growth inhibition. The inhibition ratio of EspB secretion, EspB%, for GE ranged from 82 to 100 (data not shown). The inhibitory effect of GE on secretion of EspB was confirmed by using SDS-PAGE (Fig. 1a). When bacteria were cultured in a medium without GE, EspC

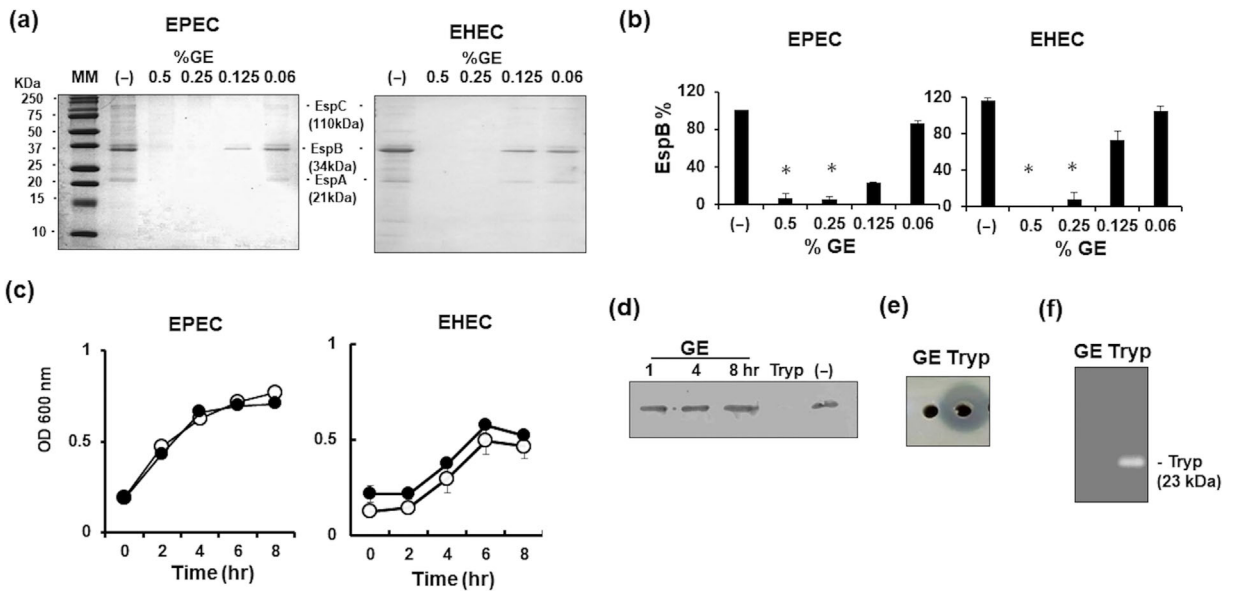

Fig. 1. Effects of *P. guajava* leaf extracts on T3SS. (a) SDS-PAGE protein staining and (b) ELISA assay of EspB of EPEC or EHEC in the culture supernatant. Bacteria were cultured in DMEM with or without (–) GE. The concentration of GE (% GE) was reduced from 0.5% to 0.06% and inhibition of EspB secretion examined. The bar graphs show the mean ELISA assay results and EspB %. Error bars, SD of triplicate samples. *; $P < 0.05$ versus control. (c) The effect of 0.25% GE on bacterial growth in medium supplemented with (●) or without (○) GE. Bacterial growth was monitored every 2 hr spectrophotometrically at OD_{600}. (d) Protease activity of 0.25% GE on EspB. Recombinant EspB was 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 no treatment (–)as a negative control. (e) Detection of protease activity by skim milk agar. Ten microliters of GE or trypsin (Tryp) was added to wells and incubated at 37°C for 20 hr. Clear zones indicate that casein has been digested. Tryp, trypsin. (f) Detection of protease activity on gelatin zymography. After electrophoresis of GE and trypsin, the gel was incubated at 37°C for 20 hr. Clear bands indicate that gelatin has been digested. Tryp, trypsin. [Color figure can be viewed at wileyonlinelibrary.com]

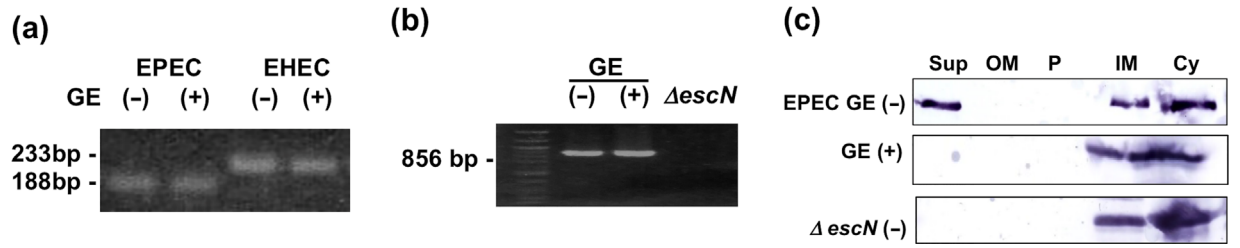

Fig. 2. Transcription of the espB gene and localization of the EspB protein. RT-PCR of (a) *espB* or (b) *escN* mRNA in bacteria grown with (+) or without (–) 0.25% GE. *espB* α (EPEC, 188 bp), *espB* γ (EHEC, 233 bp) and *escN* (856 bp) in bacterial cytosol were detected by RT-PCR. (c) EspB localization in bacteria grown with (+) or without (–) 0.25% GE. Bacteria cultured with or without GE were fractionated into culture supernatant, outer membrane, periplasmic, inner membrane and cytoplasmic fractions and EspB detected by western blotting. *ΔescN* (–), T3SS mutant strain cultivated without GE; Cy, cytoplasmic; IM, inner membrane; OM, outer membrane; P, periplasmic; Sup, culture supernatant. [Color figure can be viewed at wileyonlinelibrary.com]

(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 media containing 0.5% or 0.25% GE, indicating that GE inhibits secretion of T3SS proteins (Fig. 1a). Inhibition of EspB secretion by GE was also examined using ELISA (Fig. 1b) and it was found that the inhibitory effect of GE on EspB secretion is dose-dependent (Fig. 1a,b). GE did not affect growth of either EPEC or EHEC for up to 8 hr (Fig. 1c). Because GE might have protease activity, recombinant EspB protein was incubated with 0.25% GE for 1 to 8 hr. The results of western blotting (Fig. 1d) indicated that the EspB in the culture supernatant of EPEC is not degraded by exposure to GE. The proteolytic ability of GE against casein and gelatin was examined by using a single diffusion assay or gelatin zymography. No protease activity was detected in the GE preparation (Fig. 1e,f).

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

Regardless of the presence of 0.25% GE, bacterial RNA was harvested in the same amount with a concentration of approximately 30 μg/mL. Transcription of *espB* and *escN* genes was quantitated by RT-PCR using 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,b), indicating that the transcription activity of both *espB* and *escN* is not influenced by the presence of 0.25% GE. Corresponding amplicons were not produced by RNA samples lacking reverse transcriptase, confirming that DNA fragments containing these two genes had not contaminated the samples (data not shown). To investigate the secretion pathway through T3SS of the

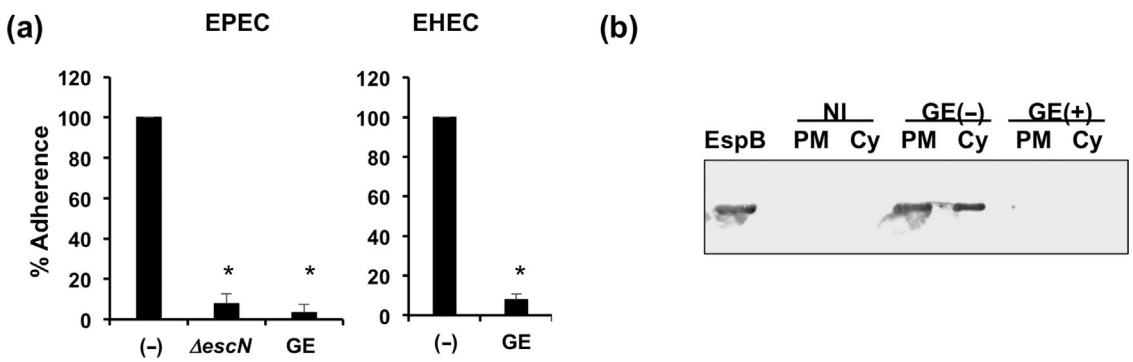

Fig. 3. Effect of 0.25% GE on bacterial adhesion and EspB injection. (a) Relative adhesion (percent ratio, related to an untreated control) of EPEC or EHEC to HEp-2 cells. Bacteria were cultured in DMEM supplemented with or without (–) GE. EPEC *ΔescN* (T3SS mutant) strain was used as a T3SS negative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

Table 1. Characteristics of pathogenic *E. coli* strains used in this study

	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 the stated locations in the stated 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* (38). (+) present, (−) absent gene.

bacteria cultured with or without GE, bacteria were fractionated into outer membrane, inner membrane, periplasmic and cytoplasmic fractions and localization of the EspB protein was examined using western blotting. As shown in Figure 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

Given that the T3SS is involved in cell adhesion, bacterial adhesion to cells may be reduced when the T3SS is not functioning. In addition, if EspB cannot be translocated into HEp-2 cells, there would be no EspB within them. To examine these matters, adhesion of the bacteria to HEp-2 cells and EspB localization within the cells were examined. Adhesion of both pathogens to HEp-2 cells was inhibited by 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 examined. When the HEp-2 cells were examined using western blotting, EspB was found in the plasma membrane and cytoplasmic fractions only in cells that had been infected with the bacteria cultured without GE (Fig. 3b).

Effects on other EPEC and EHEC strains

Additionally, othe EPEC and EHEC strain stored in our laboratory (Table 1) were tested to determine whether GE inhibits EspB secretion. It was found that GE did inhibit EspB secretion by the tested bacteria (Fig. 4a,b).

Effects on other bacterial T3SS

To test the effect of GE against other bacterial T3SS, *Y. pseudotuberculosis* and *S. Typhimurium* were examined. Secretion of the translocator proteins of *Y. pseudotuberculosis*, YopH, YopB and YopD, was

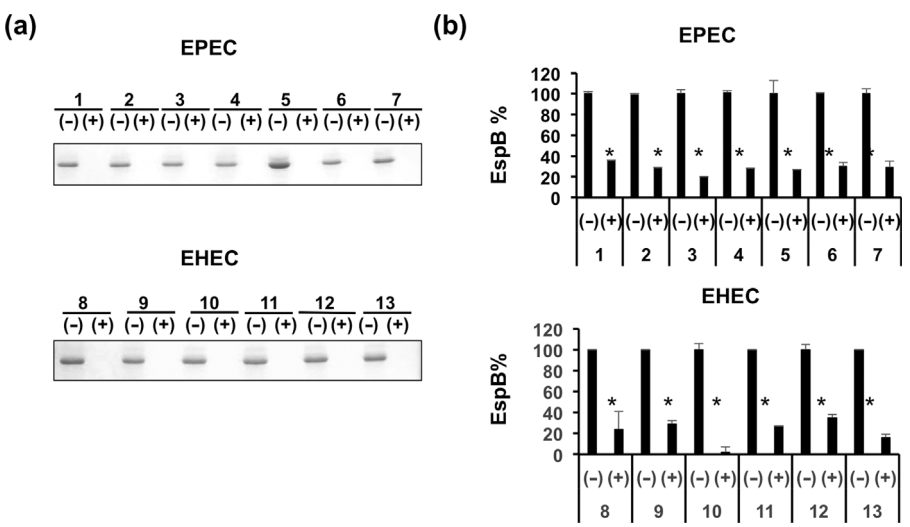

Fig. 4. Effect of 0.25% GE on other EPEC and EHEC strains. (a) Western blotting and (b) ELISA assay of the effect of GE on other EPEC and EHEC strains. The EPEC or EHEC strains listed in Table 1 were cultivated in medium with (+) or without (−) GE, after which EspB in culture supernatants was examined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

inhibited by GE without inhibition of bacterial growth (Fig. 5a,b). *Y. pseudotuberculosis* is known to induce apoptosis in macrophages via T3SS (20). Whether GE inhibits such apoptosis was therefore examined. Because the caspase cascade is induced by the apoptotic process, western blotting was used to examine caspase-3 activation. Macrophages treated with staurosporine, a reagent used to induce apoptosis, and infected with *Y. pseudotuberculosis* showed caspase-3 activation; however, macrophages infected with *Yersinia* cultured with GE did not (Fig. 5c). Apoptosis was also confirmed using the TUNEL reaction to detect the presence of DNA fragmentation (Fig. 5d). The TUNEL-positive rates were $2.6\% \pm 5.1\%$, $80\% \pm 4.4\%$ and $2.3\% \pm 0.6\%$ (mean $\pm$ SD) for no infection, infection by bacteria cultured in LB broth not supplemented with GE, and infection by bacteria cultured in LB broth supplemented with GE, respectively (Fig. 5e). In the case of *S. Typhimurium*, secretion of the T3SS translocator proteins SipB and SipC was inhibited by GE without inhibition of bacterial growth (Fig. 6a,b). *Salmonella* pathogenicity island-1 encodes T3SS and is essential for cell invasion. T3SS inhibition by GE may reduce bacterial cell invasion. Invasion of HEp-2 cells by *Salmonella* was therefore examined using a gentamycin protection assay. The invasiveness of *Salmonella* cultured with GE was reduced, ranging from 0% to 3% (Fig. 6c).

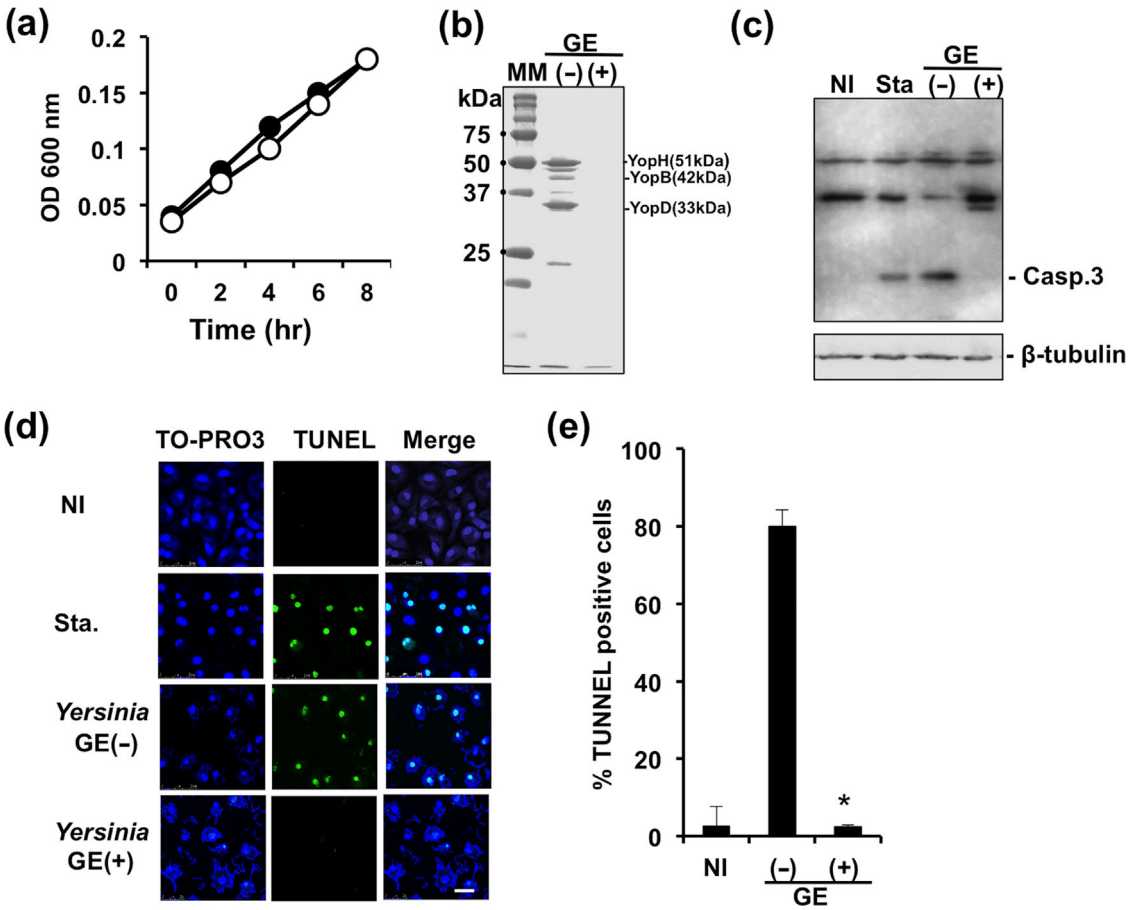

Fig. 5. Effect of 0.25% GE on *Y. pseudotuberculosis* T3SS. (a) Growth of *Y. pseudotuberculosis* cultured in LB medium with (●) or without (○) GE. Bacterial growth was monitored every 2 hr spectrophotometrically at OD_{600}. (b) Protein staining of *Y. pseudotuberculosis* T3SS proteins. *Y. pseudotuberculosis* was grown in LB broth 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) Effect of the extracts on caspase-3 activity in macrophages. Macrophages were infected with *Y. pseudotuberculosis* for 2 hr and active caspase-3 detected by western blotting. NI, not infected, Sta; staurosporine (1 μM), *Yersinia* cultured in LB broth with (+) or without (−) GE. Staurosporine-treated macrophages were used as an apoptosis positive control. (d) Induction of apoptosis in *Yersinia* infective-mouse BMMs, as determined by TUNEL assay. Sta; staurosporine (1 μM); TO-PRO3, a nucleic acid-binding dye that stains early apoptotic and necrotic cells differentially;. *Yersinia* GE (−), *Y. pseudotuberculosis* cultured without GE. *Yersinia* GE (+), *Y. pseudotuberculosis* cultured with GE. Scale bar, 10 μm. (e) Percent ratio of TUNEL-positive to total cells. The number of cells was obtained from the TUNEL image (Fig. 5d). GE (−), infection by bacteria cultured without GE, GE (+), infection by bacteria cultured with GE; NI, not infected. *, $P < 0.05$ versus without extracts.

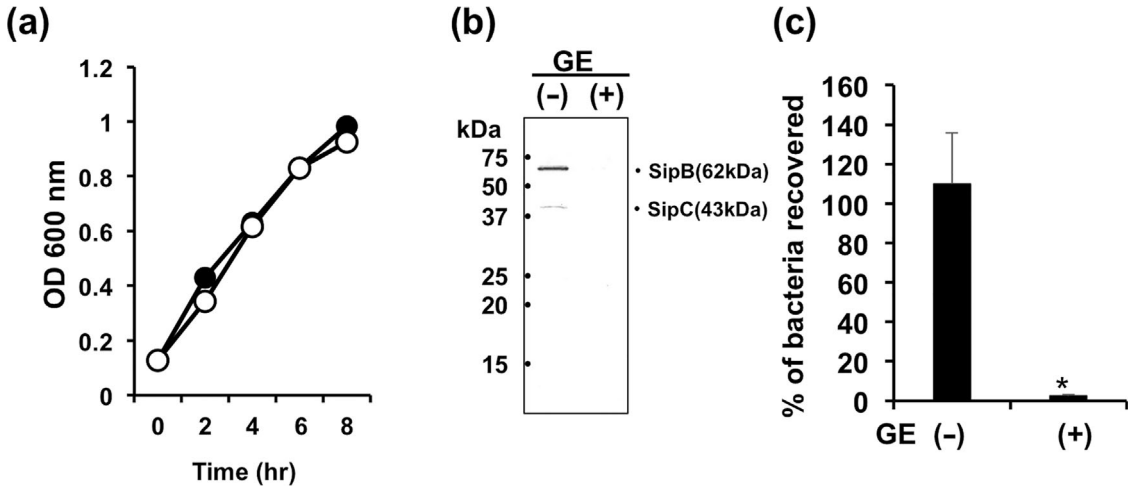

Fig. 6. Effect of 0.25% GE on *S. Typhimurium* T3SS. (a) Growth of *S. Typhimurium* cultured in LB medium with (●) or without (○) GE. Bacterial growth was monitored every 2 hr spectrophotometrically at OD_{600}. (b) Western blotting of SipB and SipC. *Salmonella* cultured in LB broth with (+) or without (−) GE. (c) Gentamycin protection assay. Macrophages were infected with *S. Typhimurium* cultured in media with (+) or without (−) GE. After killing extracellular bacteria with gentamycin, bacteria within HEp-2 cells were cultured on agar plates. Values are given as percentages of the number of bacterial colonies grown without (−) GE. $^{*}P < 0.05$ versus without extracts.

Effects of GE on other types of secretion systems

As shown in Figure 1a, a band estimated to be the EspC protein, an autotransporter protein secreted via the T5SS, was reduced in intensity in response to GE. Western blotting was therefore performed to determine the kinetics of EspC. This showed that secretion of EspC is also inhibited by GE in a dose-dependent manner (Fig. 7a), suggesting that the GE inhibits both T3SS and T5SS.

Because EHEC is known to secrete Shiga toxins (Stx1 and Stx2), which are pathogenic factors for dysentery, the effect of GE on secretion of Stx2 was investigated by western blotting. As shown in Figure 7b, GE did not affect secretion of Stx2 by EHEC, which suggests that GE affects only certain types of secretion system.

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 to attach intimately to intestinal epithelial cells and thus inject effector proteins into the host cells to cause disease (34, 35). Besides in diarrheagenic *E. coli*, T3SS is a major virulence factor in many other enteropathogenic bacteria that belong to the family *Enterobacteriaceae*, such as *Y. pseudotuberculosis* and *S. Typhimurium*. We believe that a compound that can suppress a T3SS function may have a synergic effect with already developed therapeutic compounds, including antibiotics, in cases of infectious diseases caused by T3SS-carrying pathogens.

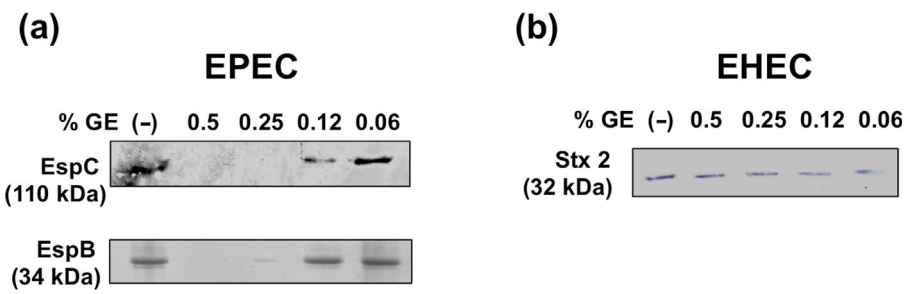

Fig. 7. Effects on other types of secretion systems. (a) Effects on EspC secretion. The culture supernatant of EPEC cultured in DMEM containing 0.5 to 0.06% GE was examined by western blot using anti-EspC anti-EspB antibodies. (b) Effects on Shiga toxin secretion. The culture supernatant of EHEC cultured in DMEM containing 0.5 to 0.06% GE was examined by western blot using anti-Stx2 antibody. [Color figure can be viewed at wileyonlinelibrary.com]

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

Next, we attempted to elucidate the mechanism by which GE inhibits the T3SS. Because Ochoa *et al.* had previously reported that lactoferrin in mammalian milk blocks 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 Figure 1d, EspB protein is not digested by GE, nor does GE digest casein or gelatin (Fig. 1e,f), suggesting that GE inhibits the T3SS in a manner that differs from that of lactoferrin. To investigate the effect of GE on the secretion machinery through the T3SS, we fractionated 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 Figure 2, we detected EspB in the inner membrane and cytoplasmic fractions regardless of whether GE was present or not. These results suggest that GE does not affect translation and accumulation of EspB protein in bacterial cells, but rather blocks translocation of EspB protein from the bacterial cytoplasm into the extracellular space. Using EPEC, EHEC, *Y. pseudotuberculosis* and *S. Typhimurium,* we also investigated whether GE inhibits the bacterial pathogenicity known to be associated with the T3SS. As shown in Figure 3a, GE inhibits adhesion of EPEC and EHEC to mammalian host cells, which we consider was mediated by blockade of T3SS. As shown in Figure 5d and e, GE inhibits apoptotic cell death induced by the T3SS of *Y. pseudotuberculosis*. As shown in Figure 6c, GE also inhibits intracellular infection by *S. Typhimurium*.

We found that GE inhibits both T3SS and T5SS (Fig. 7a). However, GE does not inhibit secretion of Stx2 (Fig. 7b). These results suggest that GE inhibits a mechanism that is common to T3SS and T5SS and is not a component of the secretion machinery of Stx.

In conclusion, GE appears to contain a novel, wide-spectrum inhibitor of the T3SS. Although the mechanism requires further elucidation, the active component of GE may be a good candidate for treating infectious diseases caused by T3SS-positive enteropathogenic bacteria.

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DISCLOSURES

No conflicts of interests are declared.

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