Inhibitory Effects of Active Compounds from *Punica granatum* Pericarp on Verocytotoxin Production by Enterohemorrhagic *Escherichia coli* O157 : H7

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***Punica granatum* (*P. granatum*) Linn. pericarp has been commonly employed as a crude drug in Thai traditional medicines for the treatment of diarrhea. The antibacterial activity of extracts from *P. granatum* pericarp against different strains of enterohemorrhagic *Escherichia coli* (*E. coli*) O157 : H7 and other strains of enterohemorrhagic *E. coli* were investigated. Successive chloroform, 95% ethanol, and water extracts of the plant were examined. The ethanolic extract was found to be the most effective against all the strains examined. Both the ethyl acetate and *n*-butanol fractions of *P. granatum* pericarp were demonstrated to have high activity with the highest minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of 0.05 and 0.05 and 0.39 and 0.19 mg/ml, respectively. The inhibitory effects of both fractions on the production of verocytotoxin (VT)1 and VT2 by enterohemorrhagic *E. coli* O157 : H7 was observed at very low concentrations of 1/10 of the MIC value (0.05–0.09 mg/ml, respectively). Phytochemical screening of the ethanolic extracts demonstrated that they contain flavonoids, sterols, triterpenes, phenols, and tannins. Our findings suggest that an appropriate bioactive compound may be developed from *P. granatum* pericarp as alternative treatment for this *E. coli* O157 : H7 infection. The crude plant may also be administered to prevent VT production in the human intestine to solve the problem of subinhibitory effects from the use of antibiotics.**

Key words —— enterohemorrhagic *Escherichia coli* O157 : H7, verocytotoxin, *Punica granatum*, medicinal plants, antibacterial activity

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INTRODUCTION

Enterohemorrhagic *Escherichia coli* (*E. coli*) O157 : H7 is of great clinical and epidemiologic importance as the etiologic agent in significant human diseases, including diarrhea,[1)] hemorrhagic colitis (HC),[2)] and occasionally complications such as hemolytic-uremic syndrome (HUS), and thrombocytopenic purpura (TTP)[3,4)] in developed countries. Two types of verocytotoxin (VT) termed VT1 and VT2 have been implicated as important factors in the mechanism of action of *E. coli* O157 : H7.[5,6)]

The interest in plants with antimicrobial properties has been revived due to current problems associated with the use of antibiotics with the increased prevalence of multiple drug-resistant strains of a number of pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus*,[7–9)] *Helicobacter pylori*,[10,11)] and multiple drug-resistant *Klebsiella pneumoniae*.[12,13)] On the other hand, infection with *E. coli* O157 : H7 involves the risk stimulation of VT production.[14,15)] Herbal remedies are viewed as a reemerging health aid in a number of countries.[16)] This can be traced to both the increasing costs of prescription drugs for the maintenance of personal health and antibiotic-resistant strains in the case of infectious diseases. In industrialized countries, the extraction and development of many drugs and chemotherapeutics from medicinal plants have been increasing.[17)] Complications resulting from the use of antibiotics in the treatment of HUS and TTP encourage researchers to find effective medicinal plants as alternative treatments for *E. coli* O157 : H7 infection.

Punica granatum (*P. granatum*) Linn., commonly known as pomegranate, granade, granats, Carthaginian apple, Punica apple or tuptim in Thai is a shrub that grows well in warm climates. The plant belongs to the family *Punicaceae* and is localized in South east Asia including Thailand. The fruit rind,[18,19)] fruit,[20)] and leaves[21)] have been commonly

Table 1. Enterohemorrhagic *E. coli* O157 : H7 Strains Used

Bacterial strain	Serotype	VT	Origin	Other features[a)]
RIMD 0509952	O157 : H7	VT 1 and VT 2	Human	Not examined
RIMD 05091055	O26 : H11	VT 1	Human	espP, hlyA, eaeA
RIMD 05091056	O111 : NM	VT 1	Human	espP, hlyA, eaeA
RIMD 05091078	O157 : H7	VT 1 and VT 2	Human	Not examined
RIMD 05091083	O157 : H7	VT 2	Human	Not examined
RIMD 05091556	O22	VT 2	Bovine	(eaeA-)

a) Identified by PCR amplification.

employed in traditional medicines for diarrhea. The efficacy of the seed as an antidiarrhea agent has also been reported.[22)]

The present communication deals specifically with a detailed study of *P. granatum* activity against three different strains of *E. coli* O157 : H7 and other strains of Shiga-like toxin producing strains including *E. coli* O026 : H11, *E. coli* O011 : NM, and *E. coli* O22. Both the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the ethyl acetate and *n*-butanol fractions from ethanolic extracts of *P. granatum* pericarp were established. Furthermore, its inhibitory activity against VT production by *E. coli* O157 : H7 was investigated.

MATERIALS AND METHODS

Plant Materials ——— The pericarp of *P. granatum* was chosen on the basis of traditional practices by Thai herbalists. Taxonomic identification of the plant was established. A classified reference voucher specimen was deposited in the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Preparation of Extracts and Isolation of Active Fractions ——— Plant material was collected and washed with distilled water. Samples were cut into small pieces and dried at 60°C overnight. The extraction procedures were described previously.[23)] The plant parts were crushed in a mechanical mortar and successively extracted with solvents of increasing polarity beginning with chloroform, ethanol, and boiling water. The solvents were removed under reduced pressure in a rotary evaporator until they became completely dry. The percentage yield for each extract was determined. For the antibacterial assay, extracts and pooled fraction residues were diluted in the corresponding extraction solvents (agar diffusion method) and in dimethyl sulfoxide (DMSO, Merck, Germany) broth dilution (dilution method).

Bacterial Strains Tested ——— Characteristics of six enterohemorrhagic *E. coli* strains collected at the Research Institute for Microbial Diseases, Osaka University, are illustrated in Table 1. *E. coli* O157 : H7 RIMD 05091078, RIMD 0509952, and RIMD 05091083 were isolated from the 1996 outbreak in Osaka Prefecture, Japan. Strain RIMD 05091078 produced both VT1 and VT2 ($VT1^+$, $VT2^+$), and RIMD 05091083 produced only VT2 ($VT2^+$). Other Shiga-like toxin-producing strains included *E. coli* O26 : H11 RIMD 05091055 ($VT1^+$), *E. coli* O111 : NM RIMD 05091056 ($VT1^+$), and *E. coli* O22 RIMD 05091556 ($VT2^+$). *E. coli* ATCC 25922 was used as a reference strain. Each bacterial strain was inoculated in Mueller-Hinton broth (MHB, Difco, France) and incubated at 37°C for 18 hr. Mueller-Hinton agar (MHA, Difco, France) was used to determine antibacterial activity.

Determination of Antibacterial Activity Using the Paper Disc Agar Diffusion Method ——— Sterile filter paper discs (Whatman No. 1; 5 mm in diameter) were soaked with 10 μl of extract residue diluted in the corresponding extraction solvent, so that each disc was impregnated with 2.5 mg of residue and dried at 37°C overnight. Discs were applied onto the surface of MHA plates seeded with a 24-hr culture of the test bacteria in Trypticase soy broth (TSB, Difco, France) and incubated at 35°C for 18 hr. Antibiotic discs containing amikacin, ampicillin, chloramphenicol, gentamicin, kanamycin, norfloxacin, and tetracycline (10–30 μg) were used as controls. Susceptibility patterns were obtained according to the National Committee for Clinical Laboratory Standards (NCCLS).[24)] The antibacterial activity was evaluated by measuring the diameter of the inhibition zone formed around the disc. The experiments were performed in triplicate and the mean diameter of the inhibition zones was calculated.

Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration —— A modified agar microdilution method[25] was used to determine the MIC of extracts of the medicinal plants that produced inhibition zones. One microliter of an overnight culture of each bacterial strain containing approximately 10^4 cells were applied onto MHA supplemented with the medicinal plant extracts. The microtiter plates were incubated at 35°C for 18 hr. Observations were performed in triplicate, and the results are expressed as the mean values of the lowest concentration of plant extracts that produced a complete suppression of colony growth (MIC). The MBC using the agar dilution method in Petri dishes with Millipore filters was performed with the extracts that showed significant MIC values against each bacterial strain.

Preparation of Culture Supernatants and Cell Extracts —— The strains were precultured in 1 ml of casamino acid yeast extract (CAYE) broth (20 g of casamino acids, 6 g of yeast extract, 2.5 g of NaCl, 8.71 g of K_2HPO_4, and 1 ml of trace salt solution per liter of distilled water; the trace salt solution was composed of 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$ dissolved in 0.0005 M H_2SO_4)[26] at 37°C overnight with rotation at 100 rpm. Ten microliters of the precultured broth was inoculated into 1 ml of fresh CAYE broth (approximately 1.00×10^7 cells/ml) with or without plant extracts. After 16-hr culture at 37°C with rotation, the culture was then centrifuged at $5000 \times g$ for 5 min to separate the supernatant and cell pellets. The cell pellets were washed three times with phosphate-buffered saline (PBS) and suspended in 0.25 ml of Tris–HCl buffer 0.01 M (pH 7.5). VT in the periplasmic space was obtained by the treatment of the cell pellets collected by the above centrifugation method after treating the pellets with polymyxin B (5000 IU/ml) at 37°C for 30 min.[27] The supernatant and cell extracts were sterilized by filtration (pore size 0.20 μm).

Reverse Passive Latex Agglutination (RPLA) Assay —— RPLA titers of VT1 and VT2 in the 16-hr culture supernatant fluid and cell-associated (periplasmic) VT1 and VT2 were separately determined using the RPLA assay kit (*E. coli* Verotoxin detection kit; Denka Seiken Co., Tokyo, Japan).[28] Twenty-five microliters of the filtrates and their two-fold serial dilutions were mixed in a 96-well v-bottomed microtiter plate (Nunc; Gibco BRL, Burlington, Ontario, Canada) with the same volume of the reaction medium containing latex beads coated on anti-VT1 antibody-sensitized latex and anti-VT2 antibody-sensitized latex, in accordance with the procedure described in the manufacturer's manual. Titers are expressed as the reciprocal of the final dilution at which agglutination with latex beads was observed after 18 hr at room temperature.

Phytochemical Screening —— Phytochemical examinations of the ethanolic extract of *P. granatum* pericarp were performed as previously described.[29,30] Shinoda test (Mg-HCl) reagent was used for flavonoids, Liebermann-Burchard reagent for steroids and triterpenes, ferric chloride reagent for phenolics and tannins, Borntrager's test for anthraquinones, and Molish test for glycosides.

RESULTS AND DISCUSSION

The crude ethanolic extract from *P. granatum* produced inhibition zones against *E. coli* O157 : H7, other enterohemorrhagic *E. coli*, and the reference strains. The antibacterial activity of the crude ethanolic extract (2.5-mg disc diffusion method) resulted in clear inhibition zones of at least 10 mm for all the strains tested (Table 2).

Significant antibacterial effects, expressed as MIC value, of the crude extracts, ethyl acetate, and *n*-butanol fractions against *E. coli* O157 : H7, *E. coli* O26 : H11, *E. coli* O111 : NM, and *E. coli* O22 are presented in Table 3. Both the ethyl acetate and *n*-butanol fractions of *P. granatum* were significantly effective against *E. coli* O157 : H7 strains with the greatest MIC value at 0.05 mg/ml. They also demonstrated potent bactericidal activity with the greatest MBC value at 0.19 mg/ml.

In addition to complications of the use of antibiotics in the treatment of HUS and TTP, problems of increased in VT production may also be encountered. Although usual doses in the actual treatment of *E. coli* O157 : H7 are higher than the MIC values, some strains may acquire resistance and thus the usual dose may become a subinhitibory concentration and cause a marked stimulation of VT production. This study is a preliminary evaluation of the antibacterial activity of medicinal plants against *E. coli* O157 : H7. This is the first report of the significant antibacterial activity of *P. granatum* against enterohemorrhagic *E. coli*. The petroleum ether extract, chloroform extract, and methanol extract of *P. granatum* showed some antibacterial activities against *E. coli*.[31]

Epigallocatechin gallate and gallocatechin gallate in green tea catechins were demonstrated to inhibit the extracellular release of VT from *E. coli*

Table 2. Antibacterial Activity of Extracts of *P. granatum* Pericarp (Concentration 2.5 mg/Disc)

Bacterial strain	Mean value of inhibition zone (mm)		
	Chloroform extract	Ethanolic extract	Aqueous extract
E. coli O157 : H7 RIMD 0509952	—	10.95+/9.80[a)]	8.25/7.00
E. coli O157 : H7 RIMD 05091078	—	12.75/11.33	7.15/6.25
E. coli O157 : H7 RIMD 05091083	—	11.23/11.05	7.10/6.25
E. coli O26 : H11 RIMD 05091055	—	11.53/11.43	6.43/6.05
E. coli O111 : NM RIMD 05091056	—	11.68/10.60	6.45/6.05
E. coli O22 RIMD 05091556	—	12.00/10.80	6.25/6.20
E. coli ATCC 25922	—	10.95/10.08	7.15/7.05

—, no inhibition zone; +, wet disc; *a*) dry disc.

Table 3. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration of Ethanolic Extracts and Active Fractions of *P. granatum* Pericarp against *E. coli*

Bacterial strain	Antibacterial activity (mg/ml)		
	Ethanolic extract	Ethyl acetate fraction	*n*-Butanol fraction
E. coli O157 : H7 (RIMD 05091078)	0.39/12.5[a)]	0.05/0.39	0.09/0.19
E. coli O157 : H7 (RIMD 05091083)	3.13/25.0	0.09/1.56	0.19/3.13
E. coli O26 : H11 (RIMD 05091055)	3.13/12.5	0.19/0.78	0.05/0.39
E. coli O111 : NM RIMD 05091056	3.13/12.5	0.39/0.78	0.39/0.39
E. coli O22 (RIMD 05091556)	1.56/12.5	0.78/6.25	0.19/1.56
E. coli ATCC 25922	0.39/6.25	0.19/3.13	0.19/6.25

a) MIC/MBC.

Table 4. Inhibitory Effects of Fractions of *P. granatum* Pericarp on VT Production by *E. coli* O157 : H7 (RIMD 05091078)

Fraction	Concentration	VT titer			
		Periplasmic space		Supernatant	
		VT1	VT2	VT1	VT2
Ethyl acetate	1/10 MIC	< 1	< 1	16	2
	MIC	< 1	< 1	16	2
	10 MIC	—	—	—	—
n-Butanol	1/10 MIC	4	16	16	2
	MIC	< 1	16	16	2
	10 MIC	—	—	—	—
E. coli O157 : H7 RIMD 05091078		16	16	16	64

—, Unreadable due to color of fractions at high concentrations. MIC of ethyl acetate fraction, 0.05 mg/ml. MIC of *n*-butanol fraction, 0.09 mg/ml.

O157 : H7.[32)] However, the ethanolic extract of *Camellia chinesis* in our study showed no activity against any strain of *E. coli* O157 : H7 examined. *Cassia alata*, another plant species, was demonstrated to have no activity against *E. coli* O157 : H7. This plant species was previously reported to have activity against *E. coli*.[33)]

In the present study, it was clear that effective fractions of *P. granatum* pericarp inhibited the production of VT in both the periplasmic space and cell supernatant (Table 4). Many factors including composition of media, aeration, and antibiotic were reported to affect the level of VT production and/or release of toxins outside the cells.[34–36)] The mechanisms involved in this process are still unclear, although active compounds may interfere with transcriptional and/or translational steps.[37)]

The antibacterial activity may be indicative of

Table 5. Phytochemical Screening of Ethanolic Extract of *P. granatum* Pericarp

Assay	Result	Inhibition of VT production
Flavonoids	+	+
Steroids & triterpenes	+	+
Phenols/tannins	+	+
Alkaloids	—	NA
Anthraquinones	—	NA
Lactone glycosides	—	NA
Iridoid glycosides	—	NA

NA, Not applicable.

the presence of metabolic toxins or broad-spectrum antibiotic compounds. *P. granatum* contains large amounts of tannin (25%). Phytochemical screening of the ethanolic extract yielded positive results for sterols, flavonoids, triterpenes, phenols, and tannins (Table 5). Antimicrobial property of tannin is well-established.[38–41] A number of interesting novel secondary metabolites such as elligitannin and punicalagin have been isolated.[7,8] The high activity of *P. granatum* against all strains of *E. coli* O157 : H7 examined, especially in terms of VT inhibition, could allow its use in the treatment of *E. coli* O157 : H7 infection.

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