Ellagitannin from *Quercus infectoria* eradicates intestinal colonization and prevents renal injuries in mice infected with *Escherichia coli* O157 : H7

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The use of antimicrobial agents in the management of patients infected with Shiga toxin-producing *Escherichia coli* (STEC) O157 : H7 remains controversial. Here, we examined the antibacterial efficacy of a natural product, ellagitannin from *Quercus infectoria* (Qi 4), against the organism in a murine model. Streptomycin-pretreated mice were orogastrically inoculated with 2×10⁹ f.u. streptomycin-resistant *E. coli* O157 : H7. The results demonstrated a stable high level of STEC present in the faeces of the infected animals. The bacterial levels in infected mice receiving Qi 4 at MIC and 2×MIC were not significantly different from those of the untreated group (t-test; P>0.05). In contrast, Qi 4 at 4×MIC significantly reduced the numbers of STEC within 2 days (t-test; 0.05>P>0.01). No viable bacteria were detected between day 5 and day 10. Similarly, at day 10, no organisms were detected from the intestines of the Qi 4-treated group, while they were recovered at levels of 10⁸–11 and 10⁵–10 f.u. g⁻¹ in the colons and caeca of the infected mice, respectively. Histopathological findings from the infected kidneys revealed a marked increase in the number of mesangial cells and mesangial matrix. Ultrastructural examination of the kidneys from the infected mice also demonstrated proliferation of mesangial cells and an increase in the mesangial matrix. Cellular injury of endothelial cells with irregular borders and cytoplasmic bleb formation were noted. In contrast, the effects were not observed in the animals treated with Qi 4. The results clearly indicated that administration of Qi 4 could effectively eradicate the colonization of STEC O157 : H7 in the intestinal tract of mice and prevent renal injury. This compound may be an alternative candidate for a therapeutic agent against infections caused by this dangerous organism.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) O157 : H7 is one of the most important food-borne pathogens. This organism is known to produce one or more Shiga toxins (STs) or Vero toxins (VTs) which may produce diarrhoea, haemorrhagic colitis and life-threatening haemolytic uraemic syndrome (HUS) in humans and animals (O’Brien & Kaper, 1998; LeBlanc, 2003). The use of antibacterial agents to improve the course of illness caused by STEC O157 : H7 remains controversial. *In vitro* experiments have indicated that certain antibiotics may increase ST production (Yoh et al., 1997, 1999) and precipitate kidney complications (Mølbak et al., 2002). In addition, the overuse of antibiotics has already given rise to antibiotic-resistant strains during the past decades (Wilkerson et al., 2004; Fincher et al., 2009). Hence, there is growing interest in developing new alternatives to control STEC O157 : H7 infections (Isogai et al., 1998; Sugita-Konishi et al., 1999; Funatogawa et al., 2002; Gagnon et al., 2006; Babiuk et al., 2008; Wu et al., 2009).

It is well documented that most medicinal plants are less active against Gram-negative bacteria than against Gram-positive bacteria (McCUTCHEON et al., 1992). A number of plant species have been shown to have antibacterial activity against STEC O157 : H7, including *Camellia sinensis* (Isogai et al., 1998; Sugita-Konishi et al., 1999), *Vaccinium macrocarpon* (Wu et al., 2009) and *Quercus infectoria* (Wiart & Kumar, 2001). Earlier work from our laboratory has reported the broad spectrum of antibacterial activity of *Q. infectoria* or nut galls (Fagaceae) against a wide range of bacteria including this organism (Voravuthikunchai et al., 2007, 2008). The

**Abbreviations:** HUS, haemolytic uraemic syndrome; Qi 4, ellagitannin from *Q. infectoria*; ST, Shiga toxin; STEC, Shiga toxin-producing *E. coli*; TEM, transmission electron microscopy; VT, Vero toxin.
galls are an excretory product caused by infection by the larvae of *Cynips galleae tinctoriae*. They have been traditionally used as astringents, antidiabetics, local anaesthetics, anti-Parkinson’s disease agents, hypoglycaemics, anti-inflammatory agents, antioxidants and anti-diarrhoeal drugs (Basri & Fan, 2005; Voravuthikunchai et al., 2007; Kaur et al., 2008). However, the active compounds of this plant and their antibacterial activities *in vivo* are still to be resolved.

In this study, we demonstrate the antibacterial activity of the active compound ellagitannin, isolated from nut galls, against STEC O157:H7 in streptomycin-treated mice. Inhibition of intestinal colonization was monitored by the recovery of viable organisms in murine faeces and intestinal tissue homogenates.

**METHODS**

*Fraction of ethanolic extract of Q. infectoria*. Crude 50% ethanolic extract was first subjected to separation by quick column chromatography using silica gel 60GF254 (Merck) and then eluted stepwise with chloroform, methanol and a linear gradient of chloroform–methanol (100:0–0:100, v/v). The eluate (250 ml) was collected in a flask. An aliquot of a quick column fraction was spotted on thin-layer chromatography on silica gel 60GF254 plates. The plates were developed with different proportions of chloroform–methanol–H2O (6.0:3.7:0.3, v/v) as a mobile phase. After air drying, the spots on the plate were located by exposure to UV light. Qi 4 (Fig. 1) was concentrated by a vacuum evaporator.

**Experimental animals.** Female 9- to 10-week-old ICR mice, weighing between 25 and 30 g, were obtained from an outbred colony maintained in the animal house of the Faculty of Science at Prince of Songkla University. The animals were divided into three groups as follows: control group, infected group and Qi 4 treatment group. Five animals in each group were placed in clean cages. Streptomycin sulfate in drinking water (5 g l⁻¹) was given *ad libitum* 3 days prior to inoculation to remove normal bacterial microbiota. The animals were fasted overnight but had free access to commercial diet and drinking water containing streptomycin sulfate throughout the experiment. Observation of any side effects or mortality was noted during the period of study. Three independent experiments were performed. All procedures and experiments involving animals in this study were reviewed and approved by the Institutional Committee for Ethical Use of Experimental Animals at Prince of Songkla University.

**Bacterial strains.** Two different strains of STEC O157:H7, strain RIMD 05091078 isolated from the Okayama Prefecture outbreak in 1996 and strain EDL 933 isolated from the 1983 outbreak in USA, were used. Both strains are streptomycin-resistant and produce both VT 1 and VT 2. They were cultured in tryptic soy broth (Oxoid) at 37 °C for 18 h.

**Challenge experiments with STEC O157:H7.** Animals in each group were orogastrically inoculated with viable cultures of different strains of STEC O157:H7 through 0.86-gauge polythene tubing (Portex) fitted to a 23-gauge needle on a 1 ml tuberculin syringe. To neutralize gastric acidity, we administered 0.2 ml sterile 5% sodium bicarbonate (Merck), followed by 0.2 ml (2 × 10⁵ c.f.u. ml⁻¹) broth culture.

**In vivo study of the effect of Qi 4 on infected mice.** To test the efficacy of Qi 4 in eradicating the colonization of STEC strains, as well as to prevent any subsequent histopathological changes in mice, the

**Fig. 1.** Qi 4 structure.
animals were inoculated with STEC O157 : H7, then after 24 h they were administered daily with 0.2 ml Qi 4 to give a final concentration of MIC (0.25 mg ml\(^{-1}\)), 2 \times MIC and 4 \times MIC for 10 days. Each mouse in the control group was given 0.2 ml sterile PBS.

Quantification of faecal and intestinal bacteria. Faecal samples from individual mice were monitored at 24 h intervals for 10 days. Freshly expressed faecal pellets (approx. 0.1 g) were suspended in 1 ml sterile normal saline. The homogenate was quantified by a loop dilution method on cefixime–potassium tellurite sorbitol MacConkey agar (Oxoid) and was incubated at 37 \(^\circ\)C for 24 h. Colourless colonies recovered on selective media were confirmed by slide agglutination with anti \textit{E. coli} O157 serum (Denka Seiken). Results are expressed as means \pm SD of five animals from three independent experiments. The detection limit was 10\(^2\) c.f.u. (g tissue\(^{-1}\)).

To enumerate the organisms in the intestinal tract, mice were killed by cervical dislocation at day 10 post-inoculation. Approximately 0.5 g of ileum, colon, caecum and kidney was aseptically removed, homogenized in 4.5 ml sterile normal saline and cultured as above. Each experiment was performed at least twice with identical results.

Histopathological examination of organs. For histological examination, specific segments (1 cm in length) of distal ileum, caecum, colon and kidney were aseptically removed and cut along the midline. Each segment was washed vigorously three times in normal saline and immersion-fixed in 10% formalin and processed by standard procedures. Sections of paraffin-embedded tissues were stained with haematoxylin and eosin and viewed by light microscopy. All sections were coded to assess histopathology without bias.

Ultrastructural examination. The tissue samples from each intestinal region were fixed by immersion in 4% paraformaldehyde buffered with 0.1 M phosphate (pH 7.3) at 4 \(^\circ\)C overnight. Fixed specimens were post-fixed in 1% osmium tetroxide in the phosphate buffer at room temperature for 4 h, followed by rinsing in 0.1 M phosphate solution. The specimens were then fixed in 2% aqueous uranyl acetate for 1 h. They were then dehydrated by means of soaking in successive ethanol series: 50 and 70% for 30 min each, 95 and 100% for 1 h each. The specimens were subsequently soaked in propylene oxide for 15 min, twice. Tissues were then infiltrated with Epon-812 resin by means of the following protocol: epoxy resin:propylene oxide (1:1) for 30 min, epoxy resin:propylene oxide (1:1). The tissue was then polymerized at 70 \(^\circ\)C for 48 h.

### Table 1. Viable STEC O157 : H7 in the intestinal tract of infected mice and treated animals receiving Qi 4 at 4\times MIC (1 mg ml\(^{-1}\)) at day 10

Limit of detection was 100 c.f.u. (g tissue\(^{-1}\)). Mean counts from groups of five animals from three independent experiments.

<table>
<thead>
<tr>
<th>Mice infected with STEC O157 : H7 (2\times10^9 c.f.u.)</th>
<th>Mean \pm SD log(_{10}) [no. of organisms (g tissue(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIMD 05091078</td>
<td>&lt;2</td>
</tr>
<tr>
<td>RIMD 05091078 and treated with Qi 4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>EDL 933</td>
<td>&lt;2</td>
</tr>
<tr>
<td>EDL 933 and treated with Qi 4</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>&lt;2</td>
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</tbody>
</table>

Fig. 3. Light microscopy examination of colons of mice infected with STEC O157 : H7 (RIMD 05091078), following treatment with Qi 4 at day 10. (a) Control mouse with PBS; (b) mouse infected with STEC O157 : H7 demonstrated no noticeable change in the mucosal surface, but had an abundance of bacterial cells (arrow); (c) mouse treated with 2\times MIC (0.5 mg ml\(^{-1}\)) of the Qi 4 fraction showed a reduction in bacterial numbers in both the lumen and mucosal surface; (d) at 4\times MIC (1 mg ml\(^{-1}\)), no organisms were detected. Original magnification: \times 200.
oxide (3:1) for 30 min, pure epoxy resin for 30 min, twice. The specimens were embedded in beam capsule size 00 and cured at 80 °C overnight. Tissue blocks were trimmed with a razor blade and sectioned with a diamond knife on an ultramicrotome (MT-XL RMC). Ultrathin sections were mounted on uncoated copper grids (EMS, 200 mesh) and allowed to dry. They were then counter-stained with uranyl acetate and lead citrate, washed gently with double distilled water and allowed to dry. The ultrastructure of the tissue sections was examined under transmission electron microscopy (TEM) (JEM-100 CX II, JEOL) at 80 kV.

RESULTS

Determination of STEC O157 : H7 in faeces

Mice orogastrically inoculated with \(2 \times 10^9\) c.f.u. of two different strains of STEC and those treated daily with Qi 4 starting at 24 h post-infection survived the infection. The numbers of STEC O157 : H7 RIMD 05091078, a representative strain, in faeces over a 10-day period after infection

Fig. 4. Light microscopy examination of caeca of mice infected with STEC O157 : H7 (RIMD 05091078) following treatment with Qi 4 at day 10. (a) Control with PBS; (b) mouse infected with STEC O157 : H7 demonstrated no noticeable change in the mucosal surface, but had an abundance of bacterial cells (arrow); (c) mouse treated with \(2 \times \text{MIC} (0.5 \text{ mg ml}^{-1})\) of the Qi 4 fraction showed a reduction in the bacterial number in both the lumen and mucosal surface; (d) at \(4 \times \text{MIC} (1 \text{ mg ml}^{-1})\), no organisms were detected. Original magnification: \(\times 400\).

Fig. 5. Light microscopy examination of a renal corpuscle of mice infected with STEC O157 : H7 (RIMD 05091078) following treatment with Qi 4 at day 10. (a) Control with PBS; (b) mouse infected with STEC O157 : H7 demonstrated mesangial proliferation with an increase in the number of mesangial cells and an increase in the mesangial matrix; (c) mouse treated with \(2 \times \text{MIC} (0.5 \text{ mg ml}^{-1})\) of the Qi 4 fraction had glomeruli that appeared normal but had a slight increase in the number of mesangial cells; (d) at \(4 \times \text{MIC} (1 \text{ mg ml}^{-1})\), the glomeruli demonstrated normal numbers of mesangial cells. C, Capillary lumen; En, endothelial cell. Arrows indicate mesangial cells. Original magnification: \(\times 1000\).
are summarized in Fig. 2. The results demonstrate a stable high level of STEC in the infected animals. With Qi 4 at MIC and $2 \times \text{MIC}$, the bacterial levels in these two groups were not significantly different from those of the infected group ($t$-test; $P>0.05$). In contrast, Qi 4 at a dose of $4 \times \text{MIC}$ significantly reduced the numbers of STEC that initially colonized the intestine of mice within 2 days ($t$-test; 0.05 $P>0.01$). No viable bacteria were detected between day 5 and day 10.

**Determination of the number of STEC O157 : H7 cells in the intestinal tract**

The patterns of colonization of STEC O157 : H7 in the small intestine, caeca and colons of mice were examined on day 10 after the infection. High numbers of both bacterial strains, RIMD 05091078 and EDL 933, were present in the colons and caeca of the infected mice (Table 1). However, the organisms were not recovered from the ileum. In the Qi 4-treated group, the numbers of bacteria in the colons, as well as in the caeca, were below the detection limit.

**Histopathological findings**

Histopathological changes, such as intestinal oedema, haemorrhage or inflammatory cell infiltration, were not observed in any parts of the intestine. Evaluation of bacterial colonization revealed large numbers of the organisms present in the luminal area of colons (Fig. 3b) and caeca (Fig. 4b), but very few pathogens were noted on the mucosal surface. The colons (Fig. 3c) and caeca (Fig. 4c) of mice that received treatment with Qi 4 at $2 \times \text{MIC}$ demonstrated relatively low numbers of organisms in the lumen. Of interest was the finding that after treatment with Qi 4 at $4 \times \text{MIC}$, no bacteria were detected in either the colons or caeca of the treated animals (Figs 3d and 4d). Kidney samples from the control mice showed normal glomeruli (Fig. 5a). Glomerular lesions with mesangial cell proliferation and an increase in the mesangial matrix were observed in all sections of kidneys removed from the infected animals (Fig. 5b). In infected mice that were treated with Qi 4 at $2 \times \text{MIC}$, though glomeruli appeared normal, a slight increase in the number of mesangial cells was still observed (Fig. 5c). In contrast, treatment of the animals with Qi 4 at $4 \times \text{MIC}$ revealed normal numbers of mesangial cells within glomeruli (Fig. 5d).

**Ultrastructural examination**

Ultrastructural examination of kidneys from mice infected with STEC O157 : H7 showed the proliferation of mesangial cells and an increase in the mesangial matrix (Fig. 6b). The endothelial cells demonstrated irregular borders with cytoplasmic bleb formation (Fig. 7b). In contrast, in the kidneys of mice infected with STEC O157 : H7 and treated with Qi 4 at $4 \times \text{MIC}$, the number of mesangial cells appeared normal (Fig. 6c). The endothelial cells that lined the capillary lumen revealed normal morphology (Fig. 7d).

**DISCUSSION**

This is the first *in vivo* study, to our knowledge, to demonstrate the antibacterial activity of Qi 4 from *Q. infectoria* against STEC O157 : H7. The results revealed that Qi 4 affected bacterial growth and prevented renal injuries in mice. Administration of Qi 4 at $4 \times \text{MIC}$ after 24 h of infection completely inhibited the colonization of the organisms in the intestine of mice within 5 days. At day 10, colonization of the intestinal tract and ultrastructural changes were rarely observed in the treated mice.
Cellular damage of the endothelial cells lining small vessels in the colons, kidneys and central nervous systems is a common histopathological finding in patients with post-diarrhoeal sequelae (Richardson et al., 1988). During the development of HUS, renal glomerular microvascular endothelial cells are the putative target of ST (Zoja & Remuzzi, 1992). The cytotoxic effect of ST on human vascular endothelial cells has been documented (Zoja & Remuzzi, 1992; Obrig et al., 1988). In this study, similar observations from TEM micrographs, such as the proliferation of mesangial cells and changes in endothelial cells due to STEC infection, were observed in the untreated animals. Infections caused by STEC do not normally lead to bacteraemia. Systemic complications like HUS are caused by ST translocated from the intestine into target organs, such as the kidney and brain. Our previous work has clearly demonstrated the inhibition effect of Qi 4 on ST production by the organisms at 20 h post-exposure (Voravuthikunchai & Suwalak, 2008). Other plant-derived compounds, such as epigallocatechin gallate and gallocatechin gallate, in green tea catechins are also reported to inhibit the extracellular release of ST from STEC O157 : H7 (Sugita-Konishi et al., 1999).

Adhesion to host cells is important in intestinal infections caused by many pathogenic Gram-negative bacteria and can be influenced by the surface hydrophobicity of the microbial cell. Though the ethanolic extract of Q. infectoria could modulate hydrophobicity characteristics on the outermost surface of the bacteria, Qi 4 was unable to produce this effect (Voravuthikunchai & Limsupan, 2006). Evidence from increased adherence to HEp-2 cells suggested that ST of the organism may promote intestinal colonization (Robinson et al., 2006). In this study, we demonstrated that very few STEC were present on the mucosal surface of all infected animals. Therefore, we hypothesize that ST produced during the course of infection may play an essential role, irrespective of intestinal colonization by the organism.

Disruption in the outer wall and cytoplasmic membranes of STEC O157 : H7 was observed when the bacterial cells were treated with the ethanolic extract of Q. infectoria (Suwalak & Voravuthikunchai, 2009). Another antibacterial mechanism of Qi 4 against the organism may be due to its ability to weaken the cell membrane by high tannic and gallic acid concentration. Antimeticillin-resistant activity of Q. infectoria (Chusri & Voravuthikunchai, 2009) and anti-STE C O157 : H7 of cranberry pomace (Vattem et al., 2004) were correlated with the high ellagic acid concentration present. Structural elucidation indicated ellagitanin (C34H28O22, molecular mass 788.57 Da) as the major component in Qi 4 (result not shown).

In conclusion, this study clearly demonstrated that administration of Qi 4 effectively reduced the colonization of STEC in the intestinal tract and prevented renal injuries in mice. While the results of this in vivo study certainly provide important new data on the beneficial action of Qi 4 on STEC, further studies are required to determine whether this agent is likely to become a novel, promising antimicrobial agent for STEC O157 : H7 infections.

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REFERENCES


