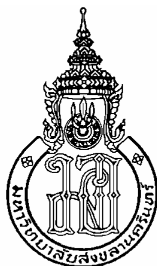

Mechanisms of Bioactive Products from *Quercus infectoria* against Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7

Sakol Suwalak

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Thesis Title Mechanisms of Bioactive Products from *Quercus infectoria* against Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7

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ชื่อวิทยานิพนธ์	กลไกของสารออกฤทธิ์ทางชีวภาพจากเบญกานีต่อเชื้อ Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7
ผู้เขียน	นายสากล สุวลักษณ์
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บทคัดย่อ

Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7 คือแบคทีเรียก่อโรคที่ปนเปื้อนในอาหารที่เป็นสาเหตุทำให้เกิดโรคท้องร่วงมีและไม่มีเลือดปนเปื้อนในอุจจาระและมีภาวะแทรกซ้อนคือการอักเสบของลำไส้ที่มีภาวะเลือดออกร่วม มักพบร่วมกับกลุ่มอาการไตชนิด haemolytic uraemic syndrome การใช้ยาปฏิชีวนะรักษาการติดเชื้อพบว่าทำให้เชื้อสร้าง Verocytotoxin (VT) และยังพบการดื้อยาปฏิชีวนะเพิ่มขึ้น ดังนั้นการศึกษาใช้สมุนไพรซึ่งเป็นยาทางเลือกจากแหล่งธรรมชาติอาจนำมาทดแทนยาปฏิชีวนะ สมุนไพรเบญกานี (nut galls) ได้ถูกนำมาทดสอบการออกฤทธิ์ต่อ *E. coli* O157: H7 และในกลุ่ม Verocytotoxin-producing enterohaemorrhagic *E. coli*

ได้ทำการทดสอบการออกฤทธิ์ของสารสกัดจากเอทานอล (ethanolic extract) จากเบญกานี ต่อเชื้อ *E. coli* O157: H7 โดยตรวจสอบการมีชีวิตของเซลล์แบคทีเรียและศึกษาการเปลี่ยนแปลงลักษณะภายนอกและภายในเซลล์โดยใช้กล้องจุลทรรศน์อิเล็กตรอน พบว่า สารสกัดจากเอทานอล สามารถยับยั้งการเจริญและฆ่าเชื้อ *E. coli* O157: H7 ทุกสายพันธุ์ที่นำมาทดสอบ มีค่าของ MICs อยู่ระหว่าง 0.78 ถึง 1.56 mg/ml และ ค่าของ MBCs อยู่ระหว่าง 1.56 ถึง 3.12 mg/ml สารสกัดจากเอทานอลที่ความเข้มข้น 4MIC ออกฤทธิ์ต่อเชื้อ *E. coli* O157: H7 พบว่าสามารถลดจำนวนเซลล์แบคทีเรียลงอย่างน้อย 2 logs ภายใน 4 ชั่วโมง และ สามารถฆ่าเชื้อ *E. coli* O157: H7 ภายใน 12 ชั่วโมง การศึกษาใช้กล้องจุลทรรศน์อิเล็กตรอนแบบลำแสงส่องกราดแสดงการเปลี่ยนแปลงที่ cell wall และรูปร่างของเซลล์แบคทีเรียที่ความเข้มข้น MIC (0.78 mg/ml) และ 2MIC (1.56 mg/ml) ตามลำดับ ในความเข้มข้น4 MIC (3.12 mg/ml) เซลล์แบคทีเรียมีรูปร่างลักษณะแฟบแบน การศึกษาโดยการใช้กล้องจุลทรรศน์อิเล็กตรอนแบบ ลำแสงส่องผ่านพบว่ามีการเปลี่ยนแปลงลักษณะภายในเซลล์แบคทีเรียที่รุนแรง ซึ่งมีความสัมพันธ์กับความเข้มข้นของสารสกัดจากเอทานอลที่สูงขึ้น เซลล์แบคทีเรียเมื่อทดสอบกับสารสกัดจากเบญกานีที่ระดับความเข้มข้น MIC พบว่า cell wall ของเซลล์แบคทีเรียโป่งพองและมี intracellular content รั่วออก

นอกเซลล์ ในความเข้มข้น 2MIC พบว่ามีการแยกชั้นระหว่างชั้น cell wall กับ cytoplasmic membrane (เยื่อหุ้มเซลล์)ให้ออกจากกัน มีการทำลายเยื่อหุ้มเซลล์บริเวณ polar ของเซลล์ ภายใน cytoplasm ของแบคทีเรียมีลักษณะ electron dense และพบช่องว่าง (vacuole) ภายในเซลล์ที่ระดับความเข้มข้น 4MIC และพบ fragmentation ของเซลล์แบคทีเรียด้วย

ตรวจสอบการออกฤทธิ์ต่อต้านแบคทีเรียของสารสกัดกึ่งบริสุทธิ์ Qi 2, Qi 3 และ Qi 4 fraction จากเบญกานีโดยใช้วิธี broth microdilution method พบว่าค่าของ MIC และ MBC มีค่าตั้งแต่ 250 ถึง 500 μg/ml ตรวจสอบการยับยั้งการสร้าง VT1 (periplasmic) และ VT2 (culture supernatant) ของเชื้อ *E. coli* O157: H7 โดยใช้วิธี immunological toxin assay และ Verotoxicity assay พบว่าสารสกัดกึ่งบริสุทธิ์ Qi 4 fraction ระดับความเข้มข้น MIC และ sub-MICs สามารถยับยั้งการสร้าง VT1 และ VT2 จากเชื้อ *E. coli* O157: H7 RIMD 05091078 ที่เวลา 20 ชั่วโมง

ศึกษาฤทธิ์ของสารสกัดจากเบญกานีต่อการเปลี่ยนแปลง cell surface hydrophobicity ของเชื้อโดยวิธี microbial adhesion to hydrocarbon (MATH) ต่อเชื้อ *E. coli* O22, *E. coli* O26: H11, *E. coli* O111: NM, *E. coli* O157: H7 และ *E. coli* ATCC 25922 พบว่าค่าเปอร์เซ็นต์ของ hydrophobicity index (HPBI) ต่อเชื้อ *E. coli* O22, *E. coli* O26: H11, *E. coli* O111: NM, *E. coli* O157: H7 และ *E. coli* ATCC 25922 ที่นำมาทดสอบมีค่าน้อยกว่า 70% ซึ่งจัดเป็นกลุ่ม hydrophilic ความเข้มข้นระดับ 4MIC(MBC) ของสารสกัดจากเบญกานีมีผลต่อการเปลี่ยนแปลง cell surface hydrophobicity ต่อเชื้อกลุ่ม *E. coli* O157: H7 พบว่าค่า hydrophobicity index (HPBI) สูงกว่ากลุ่มแบคทีเรียที่ไม่ใส่สารสกัด การเปลี่ยนแปลง cell surface hydrophobicity ของสารสกัดจากเบญกานีต่อเชื้อ *E. coli* O157: H7 ควบคุมการไม่เกิดการสัมผัสของเชื้อแบคทีเรียต่อผิวเซลล์ของ host

ศึกษาฤทธิ์ของสารสกัดกึ่งบริสุทธิ์ Qi 4 fraction จากเบญกานีในหนู (mice) ที่ได้รับเชื้อ *E. coli* O157: H7 RIMD 05091078 และ *E. coli* O157: H7 EDL 933 การศึกษานี้ทำโดยการลด normal intestinal microbiota โดยให้กินน้ำผสมยา streptomycin หลังจากนั้นหนูได้รับเชื้อ *E. coli* O157: H7 จำนวน $2x10^9$ cfu/mouse เข้าไปในกระเพาะอาหาร หลังจากหนูได้รับเชื้อ 24 ชั่วโมง หนูได้รับสารสกัดกึ่งบริสุทธิ์ Qi 4 fraction ปริมาณ 1 mg/ml ต่อวัน ตรวจนับจำนวนเชื้อ *E. coli* O157: H7 จากอุจจาระทุกวันเป็นเวลา 10 วัน ในวันที่ 10 หนูถูกฆ่า นำลำไส้เล็ก caecum และ ลำไส้ใหญ่ มาตรวจสอบนับจำนวนเชื้อ *E. coli* O157: H7 และ ศึกษาทางเนื้อเยื่อพยาธิวิทยา พบว่า จำนวนเชื้อ *E. coli* O157: H7 RIMD 05091078 และ *E. coli* O157: H7 EDL 933 ตรวจพบในอุจจาระตั้งแต่วันที่ 1 และเชื้อได้เพิ่มจำนวนขึ้นจนถึงวันที่ 10

ในกลุ่มหนูที่ได้รับเชื้อแบคทีเรีย กลุ่มหนูที่ได้รับเชื้อและสารสกัดกึ่งบริสุทธิ์ Qi 4 fraction พบว่า ในวันที่ 5 ตรวจไม่พบเชื้อ *E. coli* O157: H7 ในอุจจาระ ผลการนับจำนวนเชื้อแบคทีเรียจากเนื้อเยื่อชนิดต่างๆของหนูทุกกลุ่มในวันที่ 10 พบว่า มีจำนวนเชื้อ *E. coli* O157: H7 เฉพาะที่ caecum และลำไส้ใหญ่ เฉพาะในกลุ่มหนูที่ได้รับเชื้อแบคทีเรียอย่างเดียว

สำหรับการเปลี่ยนแปลงทางพยาธิสภาพของเนื้อเยื่อไตหนูเมื่อตรวจด้วยกล้องจุลทรรศน์ชนิดธรรมดา พบว่า เนื้อเยื่อไตหนูในกลุ่มหนูที่ได้รับเชื้อ พบว่า glomerulus มีขนาดขยายใหญ่ และมีการเพิ่มของ mesangial cells และ mesangial matrix การตรวจด้วยกล้องจุลทรรศน์อิเล็กตรอนของ glomerulus พบว่า endothelial cells มี cell membrane ไม่เรียบและโป่งพองขึ้น (irregular borders with cytoplasmic bleb formation) สำหรับกลุ่มหนูที่ได้รับเชื้อและสารสกัดกึ่งบริสุทธิ์ Qi 4 fraction ปริมาณ 1 mg/ml ต่อวัน เป็นเวลา 10 วัน ผลการตรวจทางเนื้อเยื่อพยาธิวิทยาไม่พบการเปลี่ยนแปลงใน glomerulus ของไตหนู

ผลจากการศึกษาแสดงเห็นว่าการให้สารสกัดกึ่งบริสุทธิ์ Qi 4 fraction จากเบญกานี ป้องกันการติดเชื้อ *E. coli* O157: H7 ในหนูโดย ลดการติดเชื้อ และการเจริญเติบโตของเชื้อในทางเดินอาหาร และป้องกันการเกิดพยาธิสภาพที่ไตหนูสายพันธุ์ IRC

Thesis Title Mechanisms of Bioactive Products from *Quercus infectoria* against Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7

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ABSTRACT

Enterohaemorrhagic *Escherichia coli* (EHEC) O157: H7 is one of the most important food-borne pathogens causing non-bloody and bloody diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome. The use of antibiotics has been demonstrated to result in an increased level of Verocytotoxin (VT) production as well as antibiotic-resistance. We have been studying alternative drugs from natural source. Nut galls were investigated for antibacterial activity against *E. coli* O157: H7 and other Verocytotoxin-producing enterohaemorrhagic *E. coli*.

In this study, we examined the antibacterial action of a 50% ethanolic extract of nut galls on *E. coli* O157: H7 by observing cell viability as well as morphological and ultrastructural changes using electron microscopy, and time-kill assays. The extract showed inhibitory and bactericidal effects on all strains tested with MICs of 0.78 to 1.56 mg/ml and MBCs of 1.56 to 3.12 mg/ml. Detailed studies demonstrated that numbers of viable cells treated with 4MIC of the extract decreased at least two log-fold within 4 h and were completely killed within 12 h. Scanning electron microscope illustrated a completely loss of surface appendages and pronounced at MIC and 2MIC. The whole cell appeared to have collapsed at 4MIC. Ultrastructural changes observed from transmission electron micrographs of similar samples further verified that damages in the treated cells increased with the increase in the concentrations of the extract. At MIC (0.78 mg/ml), there was some evidence that the cytoplasmic membranes of the treated *E. coli* were bulging and/or ruptured and the cells appeared to be discharging intracellular materials. At 2MIC, the outer membrane of the treated *E. coli*, which was attached to the cell wall became separated from the wall. Disruption in the cell wall and cytoplasmic membranes especially at

the polar regions of the cells occurred and some vacuolization appeared. At 4MIC, the damage to *E. coli* cells was extensive, and there was loss of their cellular integrity.

In addition the antibacterial activity of the semi-purified fractions were also described in this study. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were initially determined by a broth microdilution method. The Qi 2, Qi 3, and Qi 4 fractions from nut galls were demonstrated to possess antibacterial activity with MIC and MBC values ranging from 250 to 500 μg/ml. VT1 and VT2 activities were separately detected using an immunological toxin assay and Verotoxicity assay. An inhibitory effect of the Qi 4 fraction on VT1 and VT2 production by *E. coli* O157: H7 was observed at 20 h. The Qi 4 fraction inhibited the release of VT1 and VT2 from EHEC cells at its MIC as well as subinhibitory concentrations. These *in vitro* results clearly indicate that the Qi 4 fraction might provide promising natural alternatives to control food poisoning by *E. coli* O157: H7.

Modifications of cell surface hydrophobicity by nut galls extracts on EHEC were determined by the microbial adhesion to hydrocarbon (MATH) assay. A variety of EHEC strains isolated from disease outbreaks including *E. coli* O22, *E. coli* O26: H11, *E. coli* O111: NM, *E. coli* O157: H7, and *E. coli* ATCC 25922 were investigated. The hydrophobicity index (HPBI) of EHEC strains were found to be by far less than 70%, which indicated the hydrophilic nature of the strains. The values of HPBI of EHEC strains as well as *E. coli* ATCC 25922 at sub-MICs of the extract did not show definite patterns. At 4MIC (MBC) of the crude extract, the surface of these cells was demonstrated to exhibit a higher level of hydrophobicity towards toluene, compared with untreated cells. We therefore propose that nut galls extract modulates the first mucosal surface contact phase of infection by enhancing cell surface hydrophobicity.

The effects of the semi-purified Qi 4 fraction of nut galls was investigated in a mouse model of *E. coli* O157: H7 infection. Mice were pretreated with streptomycin, followed by an intragastrically inoculation of $2x10^9$cfu/mouse. Mice were then treated with the semi-purified Qi 4 fraction at a dose of 1 mg/ml for

ten days. The faeces were collected to determine viable cell counts of EHEC. At day ten, the mice were sacrificed, the small intestine, caecum, and colon of all mice were removed for determination of viable EHEC cells and for histopathological examination. *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933 numbers in the faeces were increased from day one to day ten. However after day five, viable bacteria were not be detected in the faeces of the treated group. At day ten, both *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933 were detected in the caecum and colon of all mice, however, bacteria were not detected in the small intestine. When the mice were treated with the semi-purified Qi 4 fraction (1 mg/ml) 24 h after the inoculation, the numbers of bacteria in the caecum as well as the colon decreased to less than 100 cfu/g faeces. The detection limit was 100 cfu/g faeces.

The histopathological finding in the kidneys infected with *E. coli* O157: H7 showed a enlarged glomerulus and a marked increase in mesangial cells, and the mesangial matrix. Ultrastructural examination of kidney from mice infected with *E. coli* O157: H7 demonstrated a marked increase in mesangial cells and the mesangial matrix, with the endothelial cells showing irregular borders with cytoplasmic bleb formation. These were not seen in the group treated with semi-purified Qi 4 fraction.

These results indicate that intragastrically administration of semi-purified Qi 4 effectively protected IRC mice against food-borne EHEC infections by preventing of renal injury and colonization of the gastrointestinal tract in mice.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	=	Percent
^{o}C	=	Degree Celsius
µg	=	Microgram
µl	=	Microlitre
AAF	=	Aggregative adherence fimbria
ADMTS	=	A disintegring and metalloprotease with thrombospondin type 1 motifs
A/E	=	Attaching and effacing
AIDA	=	Plasmidal adhesin
ATCC	=	American type culture collection
BFP	=	Bundle-forming pili
CFs	=	Fimbrial colonization factors
CDC	=	Centers for Disease Control and Prevention, USA
CT-SMAC	=	SMAC plate containing cefiximide and tellurite
CD-EC	=	Cytolethal distending toxin-producing *E. coli*
CNF	=	Cytotoxic necrotizing factor
CNS	=	Central nerve system
cfu	=	Colony forming unit
cu	=	Cured derivative
DNA	=	Deoxyribonucleic acid
DAEC	=	Diffusely adherent *E. coli*
DMSO	=	Dimethyl sulfoxide
EAF	=	EPEC adherence factor
EAST	=	Enteroaggregative heat-stable toxin
EHEC	=	Enterohaemorrhagic *E. coli*
ET	=	Electrophoretic type
EPEC	=	Enteropathogenic *E. coli*
EIEC	=	Enteroinvasive *E. coli*

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

ETEC	=	Enterotoxigenic *E. coli*
EAEC	=	Enteroaggregative *E. coli*
EspP	=	*E. coli* secreted protein protease
EIA	=	Enzyme-immunoassays
EspA	=	*E. coli* secreted protein A subunit
EDL	=	Environmental diagnostics laboratory
EtOH	=	Ethanol
EtOAc	=	Ethyl acetate
eaeA	=	Attaching and effacing gene A subunit
FFP	=	Fresh frozen plasma
FCS	=	Fetal calf serum
Gb_3	=	Globotriaosylceramide receptor
GUD	=	Glucuronidase
g	=	Gram
HC	=	Haemorrhagic colitis
HUS	=	Haemolytic uraemic syndrome
HRMEC	=	Human renal microvascular endothelial cells
HEp-2	=	Human epithelial cells
HACCP	=	Hazard analysis critical control point
HPBI	=	Hydrophobicity index
h	=	Hour
IRC	=	Institute of Cancer Research
IDDM	=	Insulin-dependent diabetes mellitus
IMS	=	Immunomagnetic separation
kDa	=	Kilo Daltal
KatP	=	Katalase peroxidase
kV	=	Kilovolte
LT	=	Heat-labile toxin
LEE	=	Locus of enterocyte effacement

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

LPS	=	Lipopolysaccharide
LM	=	Light microscope
L	=	Liter
MEM	=	Eagle's minimal essential medium
MATH	=	Microbial adhesion test to hydrocarbon assay
mRNA	=	Messenger Ribonucleic acid
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MIC	=	Minimal inhibitory concentration
MBC	=	Minimal bactericidal concentration
MeOH	=	Methanol
MHA	=	Mueller-Hinton agar
MHB	=	Mueller-Hinton broth
min	=	Minute (s)
ml	=	Millitre
mm	=	Millimetre
M	=	Molar
NSF	=	Non sorbitol fermenting
NIHID	=	National Institute of Health and Infectious Disease
nm	=	Nanometre
OD	=	Optical density
O	=	O polysaccharide, O antigen
ORF	=	Oper reading frame
OMP	=	Outer membrane protein
OsO_4	=	Osmium tetroxide crystal
PBS	=	Phosphate buffer saline
pInV	=	Invasivity plasmid
pO157	=	Plasmid pO157

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

PET	=	Plasmid-encoded enterotoxin
PCR	=	Polymerase chain reaction
PCR-RFL	=	Polymerase chain reaction-restriction fragment length polymorphism
Qi	=	Nut galls of *Quercus infectoria* fraction
RNA	=	Ribonucleic acid
RBC	=	Red blood cell
RTX	=	Repeats in toxin
RPLA	=	Reversed passive latex agglutination
RIMD	=	Research institute for microbial disease
rpm	=	Rounds per minute
rfb	=	Gene for lipopolysaccharide synthesis
SMAC	=	Sorbitol MacConkey agar
SF	=	Sorbitol fermenting
SOR	=	Sorbitor
ST	=	Heat-stable toxin
STEC	=	Shiga toxin-producing *E. coli*
Stx	=	Shiga-like toxin
stx	=	Genes for Shiga toxin
SEM	=	Scanning electron microscope
TCM	=	Traditional chinese medicine
TEM	=	Transmission electron microscope
TTSS	=	Type III secretion system
TTP	=	Thrombotic thrombocytopenic purpura
Tir	=	Translocated intimin receptor
TSA	=	Trypticase soy agar
TSB	=	Trypticase soy broth
TLC	=	Thin layer chromatography

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

UV	=	Ultraviolet
UNESCO	=	United nations educational scientific and cultural Organization
ULVWF	=	Unusually large von Willebrand factor
uidA	=	Gene for β-glucuronidase
VT	=	Verocytotoxin
VWF	=	Von Willebrand factor

CHAPTER 1

INTRODUCTION

1. Background and rationale

Enterohaemorrhagic *Escherichia coli* (EHEC), also known as Shiga toxin-producing *E. coli* (STEC) or Verocytotoxigenic *E. coli* (VTEC), is a serious pathogen causing gastrointestinal tract infections in humans. The organism is known to possess Shiga toxins (Stx) or Verocytotoxin (VT) which may cause acute diarrhoea, haemorrhagic colitis (HC) and life-threatening sequelae, haemolytic uraemic syndrome (HUS) (Leotta *et al*., 2008). The most predominant EHEC serotype responsible for disease outbreaks is O157: H7 (Tarr *et al*., 2005). Since it was recognized as a pathogen in 1982 in the United States, this serotype has been isolated in many other countries (Rivas *et al*., 2006b), and it can be associated with large outbreaks of HUS (Gobert *et al*., 2008). Among diarrhoeagenic pathogens, *E. coli* O157: H7 are described as a heterogeneous group of highly pathogenic bacteria with very low infective dose; even 1-50 organisms are capable of causing disease (Tilden *et al*., 1996).

The interest in plants with antibacterial 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 *Staphylocccus aureus* (Pesewu *et al*., 2008), *Helicobacter pylori* (Egan *et al*., 2007; Boyanova *et al*., 2009), and multiple drug-resistant *Klebsiella pneumoniae* (Nicasio *et al*., 2008; Souli *et al*., 2008). Recently, isolates of EHEC from food as well as animal faeces were reported to be resistant to cephalothin, tetracycline, and cefazolin (Srinivasan *et al*., 2007). Herbal remedies are viewed as a reemerging health aid in a number of countries (UNESCO, 1996). This is due to not only problems associated with antibiotic-resistant bacterial strains in the case of infectious diseases but also the increasing costs of prescription drugs for the maintenance of personal health. In industrialized countries, the extraction and

development of many drugs and chemotherapeutics from medicinal plants has been increasing (UNESCO, 1998). In addition, complications resulting from the use of antibiotics in the treatment of HUS (Wong *et al*., 2000; Dundas *et al*., 2001) and thrombotic thrombocytopenic purpura (TTP) are encouraging researchers to find effective medicinal plants as alternative treatments for *E. coli* O157: H7 infection.

Quercus infectoria G. Olivier (Fagaceae) (nut galls) is an evergreen shrub native of Greece, Asia Minor, and Iran. Excrescences formed upon young branches, known in market as nut galls, are the result of a puncture made in the bark by an insect, *Diplolepis gallae tinctoriae* or *Cynips quercufolii*, for the purpose of depositing its egg. The main constituents found in the nut galls are tannin (50 to 70%), and small amounts of free gallic acid and ellagic acid (Evans, 1996; Ikram and Nowshad, 1977; Wiart and Kumar, 2001). There have been many reports on nut galls activities which include molluscicidal (Redwane *et al*., 1998), larvicidal (Redwane *et al*., 2002), anti-fungal (Digraki *et al*., 1999), anti-diabetic (Hwang *et al*., 2000), anti-viral (Hussein *et al*., 2000), anti-venom (Pithayanukul *et al*., 2005), anti-inflammatory (Kaur *et al*., 2004), and its ability to act as an astringent (Muhamad and Mustafa, 1994). Though there are some studies of the activities of this plant against bacteria (Hwang *et al*., 2004; Basri and Fan, 2005; Singh *et al*., 2005; Voravuthikunchai *et al*., 2002; Voravuthikunchai *et al*., 2004; Voravuthikunchai and Limsuwan, 2006), detailed studies on antibacterial mechanisms are still limited. This study was aimed to isolate the antibacterial compounds from the nut galls against *E. coli* O157: H7 and to investigate their effectiveness as alternative treatments of *E. coli* O157: H7 infection.

2. Review of the literature

2.1 Infections caused by *E. coli*

Escherichia coli is a Gram-negative, non-sporing facultative anaerobic rod, usually motile by peritrichous flagella. It normally constitute part of the normal microbiota of the gastrointestinal tract of humans and other mammals. In clinical microbiology, *E. coli* is the most commonly isolated facultative anaerobic Gram-negative rod in faeces, and a common cause for intestinal and extra-intestinal

infections, the most common clinical outcomes of infection caused by pathogenic *E. coli* (Kaper *et al*., 2004). Thousands of serotypes of *E. coli* within the *Escherichia* family have been described. With respect to enteric diarrhoea, there is a wide spectrum of clinical presentations from a mild diarrhoea to severe, bloody diarrhoea and associated fevers that can in a small number of cases progress to more severe, and in some cases life-threatening outcomes such as HC and HUS. The clinical outcomes is dependent on the strain of *E. coli* causing the infection as will be described.

2.2 Classification of diarrhoeagenic *E. coli*

Diarrhoeagenic *E. coli* are recognized as belonging to seven major classes: enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), cytolethal distending toxin-producing *E. coli* (CD-EC), diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998; Clarke, 2001). These are shown in **Table 1.1**.

Table 1.1 Comparison of the pathogenic mechanisms of diarrhoeagenic *Escherichia coli*.

E. coli class	Main virulence factors
EAEC	Aggregative adherence fimbriae (AAF/I and AAF/III), plasmidal enterotoxin (PET), enteroaggregative heat stable toxin 1 (EAST-1)
EHEC	Verocytotoxin (VT)/Shiga-like toxin (Stx)
EIEC	Plasmid-mediate invasion genes
EPEC	Bundle-forming pili, intimin, translocated intimin receptor (Tir)
ETEC	Heat-stable (ST) and heat-stable enterotoxin (EAST 1)
CD-EC	Cytolethal distending toxin (CDT)
DAEC	α haemolysin and cytotoxic necrotizing factor 1 (CNF 1)

(from Kaper *et al*., 1997; Torres *et al*., 2005).

2.2.1 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) has been recognized as an important agent of acute and prolonged diarrhoea, especially in children of developing countries, a common cause of traveller's diarrhoea, and increasingly an agent of outbreaks in developed countries since 1987 (Huang and Dupont, 2004; Nataro, 2005). Enteroaggregative *E. coli* strains are a highly heterogeneous group consisting of about of 90 identified serotypes, the most common being O15: H18, O44: H18, O77: H18, O111: H12, O125, and O126 (Okeke and Nataro, 2001). Enteroaggregative *E. coli* are defined by their aggregation to intestinal epithelial cells in a characteristic 'stacked-brick' pattern (Nataro and Kaper, 1998). During pathogenesis, EAEC adhere to the intestinal mucosa by the plasmid encoded aggregative adherence fimbriae (AAF/I and AAF/III). This is associated with increased production of mucous biofilms, mucosal toxicity due to inflammation, and cytokine release (Huang and Dupont, 2004; Torres *et al*., 2005). Other virulence factors of EAEC include a plasmidal enterotoxin (PET) (Eslava *et al*., 1998) and an enteroaggregative heat stable toxin 1 (EAST-1) (Kuhnert *et al*., 2000; Okeke and Nataro, 2001; Zhou *et al*., 2002).

2.2.2 Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *E. coli* (EHEC) are among the leading causes of food-and water-borne illnesses affecting human in the United States, Europe, and Japan (Blanco *et al*., 2004). The term 'EHEC' was used to denote the Shiga toxin-producing *E. coli* (STEC), synonymous with the Verocytotoxigenic *E. coli* (VTEC) (Feng *et al*., 2005). The alternative nomenclature, STEC, reflects the fact that one of the cytotoxins produced by this organism is essentially identical at the genetic and protein levels to the Shiga toxin (Stx) produced by *Shigella dysenteria*e 1. The term 'VTEC' was given to strains that produced a toxin that has an irreversible cytopathic effect on Vero cells. Currently, enterohaemorrhagic *E. coli* strains belonging to 100 different O: H serotypes have been associated with human disease (Law, 2000). Common EHEC serotypes associated with human pathogenicity include O26: H11, O103: H2, O111: H8, O145: H28, O157: H7, and O157: H- (Doyle *et al*., 2001; Mora

et al., 2003). The dominant EHEC serotype associated with both outbreaks and sporadic cases of human disease that has emerged in the United States, Canada, and United Kingdom is O157: H7 (Pradel *et al*., 2000a). Enterohaemorrhagic *E. coli* O157: H7 affects people of all age groups, but the most severe infections occur amongst children and the elderly (Law, 2000). This pathogen has been implicated in many food-borne outbreaks involving HC and HUS (LeBlanc, 2003; Blaser, 2004). Enterohaemorrhagic *E. coli* O157: H7 was first isolated in 1975 from a woman having gross bloody diarrhoea (Padhye and Doyle, 1992). In 1982, HC and HUS were linked to infection with *E. coli* O157: H7, a serotype now classified as STEC (Johnson *et al*., 2006). This organism is the prototype EHEC and is often recognized as a major food-borne pathogen, implicated in worldwide illness. Close to 73,000 cases of *E. coli* O157: H7 infections are now estimated to occur annually in the United States (Rangel *et al*., 2005). The recovery period can be very long with some patients developing permanent kidney damage and approximately 250 people die each year in the United States (Nataro and Kaper, 1998). The high mortality associated with *E. coli* infections differentiates it from other types of *E. coli* like EAEC, EPEC, and ETEC (Law, 2000).

Evolutionarily, the O157: H7 serotype is a distinct clone that is only distantly related to other Stx producing EHEC strains. It is most closely related to an EPEC clone of serotype O55: H7, a non-Stx producing strain associated with infantile diarrhoea (Whittam *et al*., 1993). It was hypothesized that the new pathogen, *E. coli* O157: H7 emerged from an older version of O55: H7 **(Figure 1.1)** (Whittam *et al*., 1993; Feng *et al.,* 1998; Wick *et al*., 2005), which had already developed a mechanism for adherence to intestinal mucosal cells, when it acquired secondary virulence factors including Shiga-like cytotoxin production and plasmid-encoded adhesins through horizontal transfer and recombination (Whittam *et al*., 1993). Enterohaemorrhagic *E. coli* O157: H7 possesses biochemical markers that are significantly different from other *E. coli*. Virtually, all strains have a negative reaction for sorbitol fermentation, a positive reaction for raffinose and dulcitol fermentation, and a lack of β-glucuronidase (GUD) activity (Leclercq *et al*., 2001). However, some recent reports indicate that some strains of O157 may exhibit weak sorbitol fermentation with 24 to 48 h.

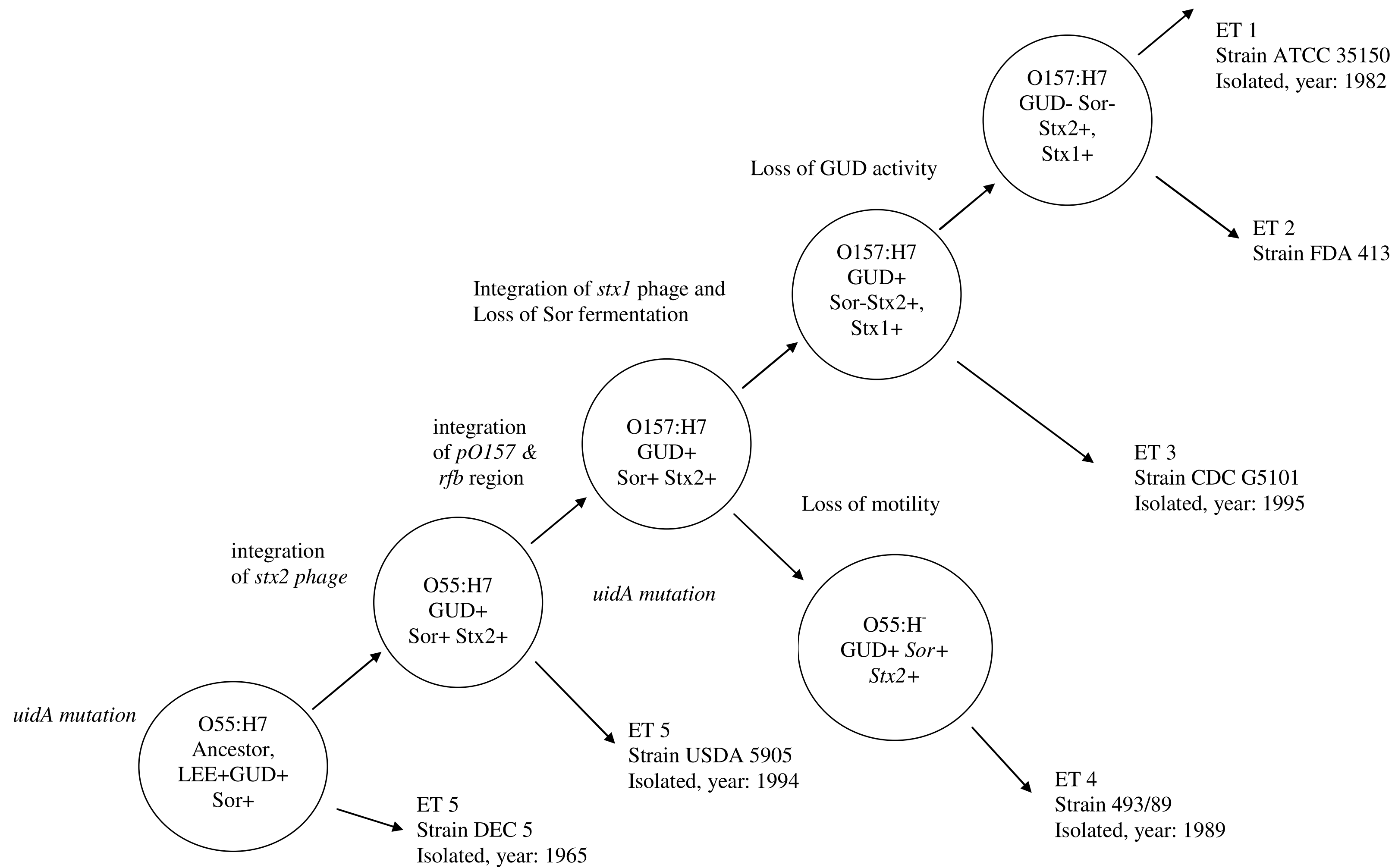


Figure 1.1 Evolution of *Escherichia coli* O157: H7 lineage modified from Whittman *et al*., 1993; Feng *et al.,* 1998; Wick *et al*., 2005.

Markers of genotypic and phenotypic variation such as *stx*, loss of sorbitol fermentation, and loss of GUD activity are typical of the common clone of *E. coli* O157: H7 found throughout the world (Kim *et al*., 2001). Molecular techniques used to detect genetic similarity among *E. coli* O157: H7 isolates from a variety of sources and geographic locations show marked homogeneity. Multilocus enzyme electrophoresis and nucleotide sequencing studies of genomic DNA support the concept of sequential emergence from a progenitor cell. These studies suggest that *E. coli* O157: H7 and *E. coli* O55: H7 originated from a progenitor cell, then acquired *stx2* prior to divergence with subsequent loss of GUD activity and sorbitol fermentation by *E. coli* O157: H7 (Feng *et al*., 1998; Tarr *et al*., 2000). The acquisition of *stx1* and other virulence factors are hypothesized to have come after acquisition of *stx2* (Shaikn and Tarr, 2003). Enterohaemorrhagic *E. coli* O157: H7 is different from other *E. coli* in terms of bacteriological, clinical, and epidemiological features. The organisms vary in size from 1 to 3 μm in length **(Figure 1.2)**.

2.2.3 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) was first described in 1944 as a paracolon bacillus and later identified as *E. coli* O124 (Lan *et al*., 2004). In 1971, EIEC strains were associated with diarrhoeal diseases (DuPont *et al*., 1971), bloody diarrhoea being typical of EIEC infection, and its occurrence has been shown to be higher in developing countries (Nataro and Kaper, 1998; Sarantuya *et al*., 2004). Enteroinvasive *E. coli* strains are generally non-motile and only a few O groups including O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, and O173 have been reported (Cheasty and Rowe, 1983; Lan *et al*., 2004). Enteroinvasive *E. coli* strains invade and multiply within enterocytes, causing the death of hosts (Escobar-Paramo *et al*., 2003; Thiem *et al*., 2004). The invasive character of EIEC is governed by a 140 MDa invasivity plasmid (pInV), which encode a type III secretion system (TTSS) and its respective TTSS effector proteins.

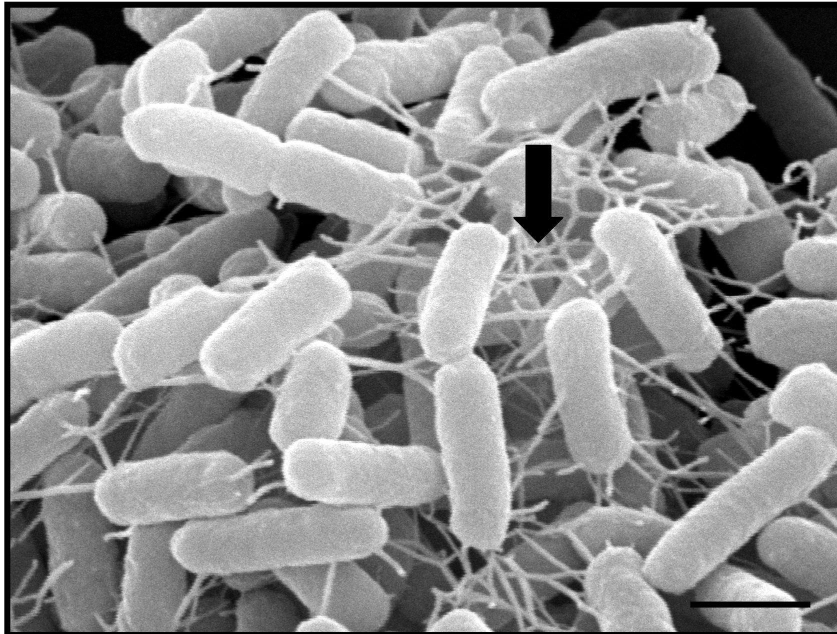


Figure 1.2 Scanning electron micrograph showing peritrichous flagella (arrow) expressed on the surface of enterohaemorrhagic *Escherichia coli* O157: H7 RIMD 05091078, Scale bar 1.5 cm = 1 µm.

2.2.4 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) was first reported in 1945 by John Bray as *Bacillus coli nepolitanum*, a causative agent of summer diarrhoea in infants (Chen and Frankel, 2005). The identification of this pathogen has previously been based on serotyping with typical O groups being O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, and O142 (Gomes *et al*., 2004). Nowadays, the hallmark of EPEC is its ability to form attaching and effacing (A/E) lesions in host cells and its lack of VT production (Chen and Frankel, 2005). Attaching and effacing lesion are characterized by destruction of microvilli, intimate adherence of bacteria to the intestinal epithelium, pedestal formation and aggregation of polarized actin, and other elements of the cytoskeleton at sites of bacterial attachments (Trabulsi *et al*., 2002). In addition, strains expressing plasmidal EPEC adherence factor (EAF) mediated by bundle-forming pili (BFP), have been defined as typical EPEC and strains lacking EAF-plasmid have been designated as atypical EPEC (Trabulsi *et al*., 2002; Chen and

Frankel, 2005). Typical EPEC, a major cause of infant diarrhoea in developing countries are rare industrialized countries where atypical EPEC seem to be a more important cause of diarrhoea (Trabulsi *et al.*, 2002). Typical EPEC strains are isolated mainly from humans, whereas atypical EPEC strains have been isolated from different animal species including cattle, sheep, and goats (Orden *et al.*, 2003; Blanco *et al.*, 2005; Cortes *et al.*, 2005).

2.2.5 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) strains were first associated with human diarrhoeal illnesses in the 1960s (Taylor *et al.*, 1961; Nataro and Kaper, 1998). Since then, the occurrence of ETEC infection has been higher in developing countries. It is also a common agent of traveller's diarrhoea and a cause for occasional outbreaks in industrialized countries (Naimi *et al.*, 2003). During pathogenesis, ETEC colonizes the intestinal epithelium using fimbriae colonization factors (CFs) and related colonization factor antigens (Kaper *et al.*, 2004). In addition, ETEC strains produce a plasmid encoded heat-stable (ST) and/or heat-labile (LT) toxin (Nataro and Kaper, 1998). The ST and LT toxins have been divided into subgroups of STa, STb and LT-I, LT-II, respectively. Of these, LT-I shares 75% amino acid similarity with cholera toxin (Nataro and Kaper, 1998; Kuhnert *et al.*, 2000). Moreover, some strains of the ETEC group have produced enteroaggregative heat stable toxin 1 (EAST-1), a toxin characteristic for the EAEC group (Savarino *et al.*, 1996).

2.2.6 Cytolethal distending toxin-producing *E. coli* (CD-EC)

Cytolethal distending toxin-producing *E. coli* (CD-EC) has been designated as an enteric pathogen, however, there is conflicting evidence for its pathogenesis (Clarke, 2001). In models examining pathogenicity, the CDT toxin has caused cell cycle arrest and subsequent death in eukaryotic cells (Clarke, 2001; Bielaszewska *et al.*, 2005). However, other diarrhoeagenic *E. coli* strains including EPEC, EAEC (Clarke, 2001; Clarke *et al.*, 2002), and *E. coli* O157: H^- strains (Janka

et al., 2003) have also been shown to produce this toxin. In contrast, CDT has rarely been characteristic for strains of *E. coli* O157: H7 (Janka *et al*., 2003).

2.2.7 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* (DAEC), also known as diarrhoea-associated haemolytic *E. coli*, have caused diarrhoea, particularly in children less than 12 months-old and it has been an important pathogen in developed countries (Nataro and Kaper, 1998; Clarke, 2001; Kaper *et al*., 2004). Diffusely adherent *E. coli* strains are characterized by their diffused adherence pattern on epithelial cells, production of α-haemolysin, and cytotoxic necrotizing factor 1 (Clarke, 2001). In addition, fimbriae adhesion F1845, mediated by both the chromosome and plasmids and plasmid-encoded adhesion (AIDA-1) have been involved in the diffuse adherence phenotype (Kaper *et al*., 2004; Torres *et al*., 2005).

2.3 Epidemiology of EHEC infection

Cattle appear to be the main reservoir of EHEC strains as they can carry this bacterium without showing any symptoms. These strains, along with STEC, can be recovered from the faecal samples of 10% to 20% of healthy cattle in the United States and Europe (Pradel *et al*., 2000b). In a recent Spanish survey, as many as 37% of the samples obtained from cattle faeces contained STEC strains (Pradel *et al*., 2000b). Enterohaemorrhagic *E. coli* O157: H7 colonizes the healthy cattle intestine, but it has also been isolated from deer, sheep, goats, horses, birds, flies, and to a lesser degree from chickens, cats, and dogs (Meng *et al*., 2001). It is found in manure, water troughs, and other places in farms, which may explain the increased risk of infection observed in people living in rural areas.

Most EHEC infections are caused by ingestion of contaminated food or water, via animal contact (Beutin, 2006), or occasionally through occupational exposure **(Figure 1.3)**. Most food-borne outbreaks have been attributed to cattle-derived foods, in particular ground beef (Voetsch *et al*., 2007), high acid foods like unpasteurized apple cider (Williams *et al*., 2004), fermented sausage (Porto-Fett *et al*.,

2008), mayonnaise (Yu *et al*., 2006), salad dressing (Beuchat *et al*., 2006), raw milk (CDC, 2008), fruits and vegetables (Franz and van Bruggen, 2008). Contaminated foods from other sources, such as lamb and jerky, have been involved in some cases (Keene *et al*., 1997). Meat probably becomes contaminated at the time of slaughter, with microorganisms internalized during grinding which may render it more likely to survive cooking. Fruits and vegetables may also be contaminated as they are often fertilized with cattle manure. Radish sprouts (Weagant and Bound, 2001), lettuce, and alfalfa sprouts (Erickson and Doyle, 2007) were implicated in several outbreaks. Radish sprouts were implicated in several outbreaks in Japan, including the massive Sakai city outbreak in 1996, which affected more than 6,000 school children (NIHIDCD, 1996). The primary source of cross-contamination is contact of food with meat or faeces contaminated with *E. coli* O157: H7 (Meng *et al*., 2001). Transmission can also occur through drinking contaminated water or exposure to contaminated recreational water reservoirs (Olsen *et al*., 2002; Muniesa *et al*., 2006). Another mode of transmission for *E. coli* O157: H7 is person-to-person transmission via the faecal-oral route. Person-to-person transmission has been reported in day care and chronic care facilities (Gomez *et al*., 2005; Miliwebsky *et al*., 2007), and also in nursing homes (Reiss *et al*., 2006). Enterohaemorrhagic *E. coli* cells have a low infective dose (Nataro and Kaper, 1998). Epidemiological data show that as few as 10 to 100 cells of *E. coli* O157: H7 per gram of raw ground beef are sufficient to cause illness.

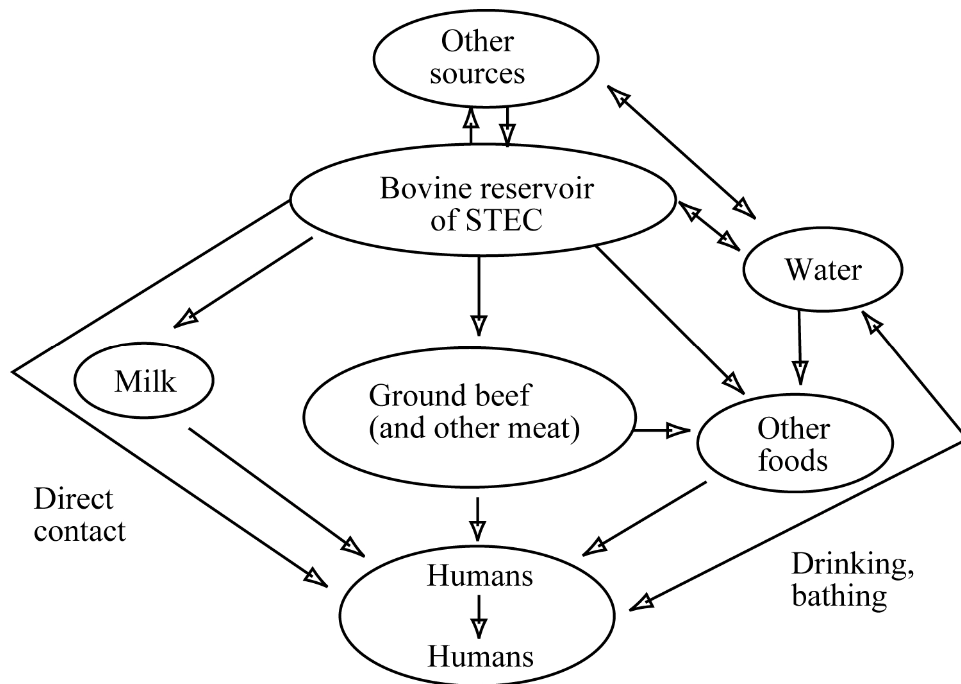


Figure 1.3 The central role of cattle in transmission of Shiga toxin-producing *Escherichia coli* (STEC) to humans. Cattle constitute a large reservoir of STEC, which may be transmitted to humans through consumption of meat and milk, direct contact with cattle, consumption of water or foods contaminated with cattle manure, or bathing in contaminated water. Ground beef is a frequent source of human disease due to O157: H7 STEC. The STEC population in cattle may be passed on to other sources such as birds and may also contain STEC strains that originated from other animal species. Infected humans may transfer the organisms to other humans (from Gyles, 2007).

2.4 Pathogenesis and virulence characteristics of typical EHEC

The production of Shiga toxins is one of the defining characteristics of EHEC as these toxins are thought to be responsible for the principal manifestations of HC and HUS (Law, 2000). However, the mechanism of pathogenesis of EHEC has not been fully elucidated (Meng *et al*., 2001). Pathogenesis of EHEC infection is a multistep process involving a complex interaction between a range of bacteria and host interactions. The main virulence factors and defining characteristics of EHEC include its ability to attach and efface intestinal mucosal cells and the production of two cytotoxins, Stx1 and Stx2 (Gyles, 2007). These toxins show a high degree of homology with Shiga toxins produced by *Shigella dysenteriae*. Although Stx1 and Stx2 are most often implicated in human illnesses, several variants of Stx2 exist. Enterohaemorrhagic *E. coli* cells remain in the intestine and Stx produced in the lumen must be first absorbed by the intestinal epithelium and translocated into the blood stream. This permits delivery of the toxin to the specific toxin receptors on target cell surfaces, inducing both local and systemic effects (Paton and Paton, 1998). It is generally considered that *E. coli* O157: H7 is more virulent than other EHEC serotypes. The most important virulence factors for *E. coli* O157: H7 are the production of Stx2 and the adhesion intimin (Law, 2000) (**Table 1.2**). Other virulence factors such as enterohaemolysin, a serine protease (EspP), and a catalase/peroxidase (Katp) may have a minor role in infection (Law, 2000). The 60-MDa plasmid present in the majority of *E. coli* O157: H7 isolates is designated as pO157. This plasmid has been sequenced and several potential virulence factors have been identified.

2.4.1 Colonization of the gut by epithelial cell adherence

After surviving the highly acidic pH in the stomach, the organism must colonize the intestine by adhering to the intestinal epithelial cells. The ability of the cells to adhere to the intestinal epithelial cells, and thus colonize the intestine is one of the key determinants of virulence (Paton and Paton, 1998).

Table 1.2 Significant virulence factors of enterohaemoharrgic *Escherichia coli* O157: H7.

Genetic Locus	Protein description	Gene	Function
Chromosome (Locus of enterocyte effacement)	Intimin	*eae*	Adherence
	Tir	*tir*	Intimin receptor
	Secretion proteins	*esp A*, *esp B*, *esp D*	Induces signal transduction
	Type III secretion system (TTSS)	*esc C, esc D, esc F, esc R, esc S, esc T, esc U, esc V, esc Q, esc Z*	Apparatus for extracellular protein secretion
Phage	Shiga toxin	*stx1, stx2, stx2c, stx2d*	Inhibits protein synthesis
Plasmid	EHEC haemolysin	*EHEC-hly A*	Disrupts cell Membrane Permeability
Plasmid	Catalase-peroxidase	*Kat P*	Disrupts cell Membrane permeability

(from Meng *et al*., 2001).

The processes involved in the establishment and maintenance of gut colonization by EHEC are poorly understood. Within the *E. coil* O157: H7 strains, there is a great heterogeneity in adherence patterns, which may reflect different mechanisms. Strains may adhere in a diffused fashion, or have localized adherence (from tight clusters or micro colonies at a limited number of sites on the epithelial surface) or may form a distinct pattern of adherence called 'log jam', in which adherence occurs principally at junctions between cells (Paton and Paton, 1998). Adherence to intestinal epithelial cells is an early feature of EHEC infection and has been investigated primary through the use of cultured cell lines of various origins and in *in vivo* studies. A more detailed explanation of the genes and mechanisms involved in attachment will be discussed in the following section.

2.4.2 Other adherence mechanisms

There are some factors that have been shown to affect the adherence of EHEC cells to host epithelial cells including factors present in whole cells, outer membrane proteins (OMPs) and lipopolysaccharides (LPS). Antibodies prepared against whole cells and outer membrane proteins, including a 94 kDa OMP and an 8 kDa OMP, but not to H7 flagella antigen, were found to significantly inhibit the adherence of *E. coli* O157: H7 to HEp-2 cells. This LPS enhances the cytotoxicity of Stx on human vascular endothelial cells *in vitro*, but its effects *in vivo* are not clear. Oelschlaeger *et al*., (1994) stated that *E. coli* O157: H7 can invade cultured intestinal cell lines, but a later report (McKee *et al*., 1995) disputed these findings, showing that *E. coli* O157: H7 strains were no more invasive than *E. coli* strains from the normal intestinal microbiota. Furthermore, there is no *in vivo* evidence that invasion occurs in humans or in animals. Two other putative EHEC virulence factors have been described. These are a serine protease, EspP, which can cleave human coagulation factor V, and a bifunctional catalase peroxidase, KatP (Pradel *et al*., 2000b). However, there is no experimental proof for any role of these factors in the virulence of EHEC.

2.4.3 Adhesin molecule intimin

As mentioned above, LEE contains *eae* gene which encodes for intimin and a homologue for the receptor of intimin, ‘translocated intimin receptor’ Tir homologue (Paton and Paton, 1998). As a result of the attachment of the bacteria to the epithelial cell surface, the cytoskeleton component beneath the adherent bacteria is altered leading to the formation of a pedestal-like structure that can extend to a pseudopod. The interaction between the bacterial adhesin molecule, intimin, and its receptor in the host cell membrane, Tir, is essential for this pedestal formation. Intimin is a member of the invasion/intimin-like protein family. Donnenberg and Kaper, (1991) showed that intimin mutants could activate signaling in host cells, inducing tyrosine phosphorylation of Tir, and generalized actin accumulation, but they could not focus actin into pedestal-like structures. Further studies have shown that the patterns of attachment and interaction between EHEC and epithelial cells are markedly different in *eae*-positive and *eae*-nagative EHEC. Only *eae*-positive EHEC form A/E lesion on intestinal epithelial cells (Kaper *et al*., 2004). Although A/E lesion is not essential for bloody diarrhoea and HUS in humans, the vast majority of strains implicated in these syndromes are *eae*-positive. Thus, most EHEC are *eae*-positive, and *eae* has been identified as a risk factor for HUS (Ethelberg *et al*., 2004). However, as this is not absolute, it has led to speculation that A/E lesion might not be essential for the development of severe disease and addition factors might be involved (Pradel *et al*., 2000a).

2.4.4 Translocated intimin receptor

Attaching and effacing lesions are mediated by bacterial-host cell interactions including the trigger *in vitro* of host signal transduction pathways (Finlay *et al*., 1992). The initial localized adherence of the bacterial cells to the epithelial cells is mediated by a plasmid-encoded, bundle-forming pilus (BFP) (Donnenberg *et al*., 1992), followed by the insertion of Tir into the host plasma membrane (Kenny and Finlay, 1997). Enteropathogenic *E. coli* secretes Tir as a 78 kDa protein using the TTSS system (de Grado *et al*., 1999). After insertion into the host cell, serine,

threonine, and tyrosine are phosphorylated and undergoe an electrophoretic mobility shift to a molecular weight of 90 kDa. Translocated intimin receptor requires secreted proteins EspA, EspB and TTSS apparatus for its translocation. Translocated intimin receptor is generally divergent between EPEC and EHEC, particularly in the C terminus of the protein. C terminal tyrosine that is required to be phosphorylated for pedestal formation in EPEC is not present in EHEC. The A/E lesion formation in EHEC occurs independently of tyrosine phosphorylation. These differences that allow EHEC to from pedestals in the absence of Tir tyrosine phosphorylation have not been characterized (Goosney *et al*., 2000).

Translocated intimin receptor has three main functions that have been identified. It is translocated into the epithelial cell membrane and serves as a cell surface receptor intimin. It also nucleates actin after intimin binding. By focusing actin, it is believed that Tir acts as a bridge connecting intimin to the host cytoskeleton. Another function of Tir is that it transmits additional signals to host cells once Tir-intimin interaction occurs. These events trigger tyrosine phosphorylation of the phospholipase Cγ and other host proteins, resulting in Tir phosphorylation and other early signaling events (Abe *et al*., 1998). Translocated intimin receptor is a unique molecule as it is a prokaryotic protein that is inserted into a eukaryotic membrane, where it undergoes modifications, acts as an intimin receptor, and induces drastic cellular changes (de Grado *et al*., 1999).

2.4.5 Type III secretion system (TTSS)

As mentioned previously, LEE also encodes for TTSS which has been found in both EHEC and EPEC. Type III secretion system is made up of proteins in the bacterial inner and outer membranes and a needle complex that allows injection of effector proteins including EspA, EspB, EspD, and Tir through host cell membrane **(Figure 1.4)**. In Shiga toxin-producing *E. coli*, the needle complex consists of a short EscF needle elongated by a filament made up of EspA (Ebel *et al*., 1998). After TTSS mediated secretion, EspB and EspD proteins are thought to be incorporated into host cell cytoplasmic membrane where they form a pore ‘translocon’. Type III secretion of Tir through EspA filament and EspB-EspD pore leads to intimate

attachment of bacteria through intimin-Tir interaction. Continued production of effector proteins then leads to A/E lesions characterized by host cell actin rearrangement. *Escherichia E. coli* secreted protein A filaments are produced transiently during this process and it is speculated that the shortening or loss of these structures allows intimate attachment to occur (Knutton *et al*., 1998).

2.4.6 Shiga toxin (Stx)

The major virulence factor and defining characteristic of EHEC is the production of Stx. O'Brien and Holmes, (1987) were the first to report that EHEC produce Stx. Shiga toxin family contains two major immunologically non-cross reactive groups called 'Stx1' and 'Stx2' (O'Brien and Holmes, 1987; Nataro and Kaper, 1998). Even though they are immunologically distinct, Stx1 and Stx2 share approximately 60% DNA and amino acid homology (Law, 2000). A single EHEC strain may express Stx1 only, Stx2 only, both toxins or multiple forms of Stx2. The Stx1 from EHEC is identical to Shiga-toxin from *Shigella dysenterae* 1. While Stx1 is homogenus, there are many variants of Stx2. Many isolates can produce two or more forms of Stx2 (Schmitt *et al*., 1991). The different variants of Stx2 are designated as Stx2c (Schmitt *et al*., 1991), Stx2v, Stx2vhb, Stx2d (Pierard *et al*., 1998), Stx2e (Sonntag *et al*., 2005), and Stx2f (Schmidt *et al*., 2000). Shiga toxins 1 (Stx1) from some strains of EHEC may differ from Stx in one residue, while Stx from other strains show no sequence variation (O'Brien *et al*., 1992). Most isolates of *E. coli* O157: H7 produce Stx2 (O'Brien *et al*., 1992). Both toxins are compound toxins, composed of a single 32-kDa A subunit and a pentameric B subunit composed of 7.7 kDa monomers (Olsnes *et al*., 1981). The A subunit of the Stx family is activated by proteolytic processing and it is proteolytically nicked to yield a 28 kDa peptide A_1 and a 4 kDa peptide A_2 (O'Brien *et al*, 1992). These peptides remain linked by a disulphide bond. The A_1 peptide contains enzymatic activity while the A_2 peptide serves to bind the A subunit to the B subunits.

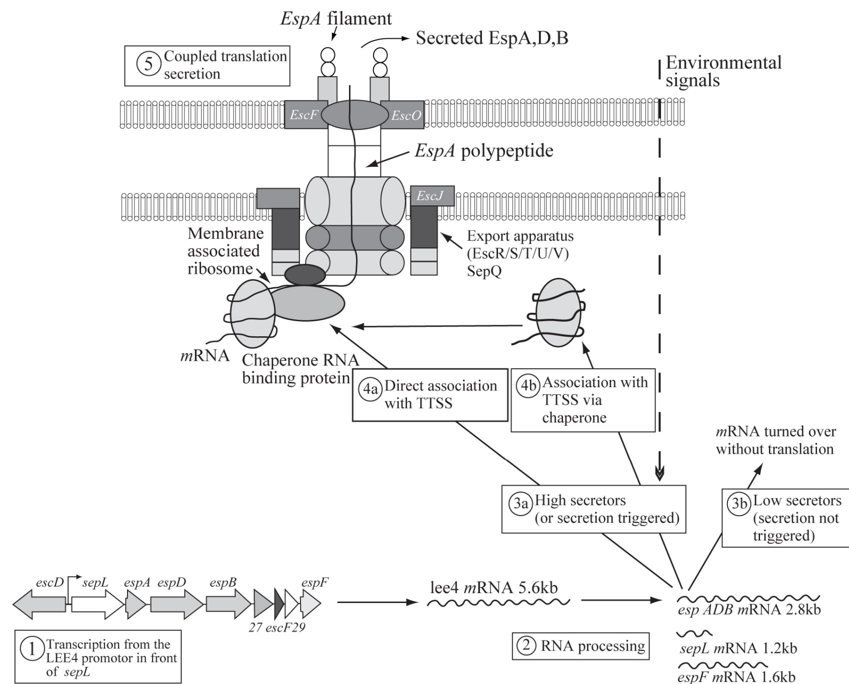


Figure 1.4 Model of *LEE4* expression and Esp protein secretion in EHEC O157 (from Roe *et al*., 2003).

(1) Transcription of *LEE4* occurs from a promoter in front of *sepL* which produces a 5.6 kb mRNA containing *espADB*. (2) This is immediately processed to produce at least three mRNA species including a 1.2 kb transcript that encodes *sepL* and 2.8 kb transcript that encodes *espADB*. (3) Processing of the 2.8 kb transcript is a key step in determining whether or not secretion occurs. (3a) Under appropriate environmental conditions, such as growth in Hepes-buffered minimal essential medium for high secretors or cell contact for low secretors, the transcript is translated at the TTSS. (3b) When secretion is not triggered, for example low secretors grown in Hepes-buffered minimal essential medium, the mRNA is still produced, but is not efficiently translated at the TTSS. It is proposed that a pool of this mRNA is present in the cell and allows immediate secretion of EspA, B and D under the right conditions, such as cell contact. (4) The mRNA may either associate directly with the TTSS (4a) or be trafficked to the TTSS via a chaperone (4b). (5) The final step is the coupled translation and secretion of the Esps at the TTSS. The effective export of at least EspD requires a chaperone, CesD also located in the membrane, presumably within the apparatus.

The B pentamer mediates binding of the toxin to specific glycolipid receptors, known as globotriaosylceramide or Gb_3, which are present on the surface of eukaryotic cell membranes (Lingwood, 2003). While Gb_3 is the main receptor for Stx, the receptor for the Stx2 variant, Stx2e, is Gb_4. The structural genes for Stx1 and Stx2 are found on lysogenic lambdoid bacteriophages (Nataro and Kaper, 1998). Production of Stx is essential for many of the pathological features and life threatening consequences of EHEC infection (Nataro and Kaper, 1998). The ability to produce Stx was acquired by *E. coli* from a bacteriophage, presumable directly or indirectly from *Shigella* (Pradel *et al*., 2000b). The expression of the phage-encoded Stx is under the regulatory control of the phage late genes. Induction of the phage lytic cycle is required for toxin synthesis and release (Muniesa *et al*., 2004; Garcia-Aljaro *et al*., 2009). Phage production is increased by factors such as antibiotic treatment or peroxide released from activated neutrophils, and these factors can also induce Stx2 production. Non-pathogenic *E. coli* can produce Stx2 if infected with the phage encoding the toxin (Muniesa *et al*., 2004; Garcia-Aljaro *et al*., 2009).

Enterohaemorrhagic *E. coli* strains appear to be unable to invade gut epithelial cells to any significant extent. Hence, the generation of systemic sequelae must presumably involve translocation of Stx by colonizing bacteria from the gut lumen to underlying tissues and the blood stream (Paton and Paton, 1998). One possible route for translocation might be through the lesions in the mucosal barrier caused either by the direct effects of Stx or other factors such as intimin or perhaps through gaps between adjacent epithelial cells. An alternative route from the gut lumen to tissues might be through intact epithelial cells (Paton and Paton, 1998). After crossing the epithelial barrier and entering the blood stream, Stx targets the tissues expressing the appropriate glycolipid receptor. The specificity of this interaction and the distribution of receptors among various cell types have a major impact on the pathogenesis of the disease (Paton and Paton, 1998). After binding to the receptors, the toxin molecules are endocytosed by a receptor-mediated endocytic mechanism and are transported to the golgi apparatus and then to the endoplasmic reticulum (Sandvig and van Deurs, 1996). The A subunit is translocated to the cytoplasm where the A_1 subunit cleaves a specific linkage in the 28S rRNA. This cleavage prevents the binding of the amino acyl tRNA to the 60S ribosomal units,

resulting in the inhibition of peptide chain elongation during protein synthesis and leads to cell death (Brown *et al*., 1981; Sandvig and van Deurs, 2001). Epidemiological evidence indicates that EHEC isolates producing Stx2 are more commonly associated with serious disease than isolates producing Stx1, or Stx1 and Stx2 (Boerlin *et al*., 1999). The toxicity of Stx2 towards human renal microvascular endothelial cells (HRMEC) is 1000 fold greater than that of Stx1 (Louise and Obrig, 1995). These cells are the putative target of the Stx in the development of HUS (Louise and Obrig, 1991; Louise and Obrig, 1995). Jacewicz *et al*., (1999) demonstrated that even though Stx1 had higher binding affinity for the Gb_3 receptor on the human intestinal microvascular endothelial cells (HIMEC) than Stx2, HIMEC were more sensitive to inhibition of protein synthesis by Stx2 than Stx1. It was concluded that increased toxicity of Stx2 to endothelial cells may be relevant to the higher frequency of Stx2 producing EHEC strains involved in the pathogenesis of HUS. In rabbit and piglet studies, the presence or absence of Stx also made no difference, further highlighting the limitation of animal models of this pathogen.

2.4.7 α-Haemolysin

The haemolytic activity of *E. coli* was first reported by Kayser in 1903, who found that some *E. coli* cultures lysed erythrocytes (Cavalieri *et al*., 1984). In 1963, Smith was first to differentiate between cell-bound and cell-free haemolysin in cultures of *E. coli* grown in alkaline meat extract broth. Under the same growth conditions, some haemolytic strains of *E. coli* can produce cell-free and cell-bound haemolysin simultaneously. The cell-free haemolytic factor was designated as α-haemolysin, as it can be obtained free from bacterial cells in culture fluid filtrates, and the cell-bound factor was designated as β-haemolysin (Beutin, 1991). A third type of haemolysin is γ-haemolysin which does not haemolyze human or rabbit RBCs, but does haemolyze RBCs of other species (Cavalieri *et al*., 1984). Enterohaemolysin is another kind of haemolysin which is also active in cell free extracts (Beutin, 1991).

Several studies have shown that haemolytic *E. coli* are more frequently isolated from extra intestinal infections such as urinary tract infections, bacteremia, peritonitis, and appendicitis than from the faeces of healthy individuals.

Other studies have also indicated that colonization before the development of infection may be enhanced by haemolysin production (Cavalieri *et al*., 1984). Haemolysin production alone does not always equate with virulence, but may be a decisive factor in virulence of many the nephrohaemorrhagic *E. coli*. *Hly*, a member of the repeat in toxin (RTX) family of pore forming cytolysins, has been suspected to have a role in pathogenesis. This is because it occurs in the majority of the pathogenic EHEC strains tested and it is reactive to sera of HUS patients (Pradel *et al*., 2000a).

2.4.8 pO157

All strains of O157: H7 contain a highly conserved plasmid, designated as 'pO157' (Schmidt *et al*., 1994) which varies in size from 93.6 to 104 kb (Schmidt *et al*., 1996). This plasmid is also present in O26: H11 strains and is present in most but not all Stx-producing *E. coli* strains isolated from humans (Levine, 1987). A 3.4-kb fragment of this plasmid, subsequently shown to encode enterohaemolysin (Schmidt *et al*., 1995), was identified by Levine in 1987 as a diagnostic probe for EHEC. In addition to enterohaemolysin and potential adherence factors described above, this plasmid encodes a catalase-peroxidase, whose function is unknown.

The role of this plasmid in EHEC pathogenesis is unknown. *In vivo* and *in vitro* studies have reported conflicting results regarding its role in adherence to epithelial cells. Karch *et al*., (1987) first reported that pO157 was required for the expression of fimbriae and adhesion to Henle 407, but not Hep-2 cells. Other investigators have reported that the loss of this plasmid either enhanced adhesion (Junkins and Doyle, 1989), decreased adhesion (Toth *et al*., 1990), or had no effect on adhesion (Fratamico *et al*., 1993). Hall *et al*., (1990) reported that for EHEC strain of serotype O103:H2, loss of this plasmid coincided with reduced adhesion to cultured epithelial cells while for another EHEC strain serotype O5: H^{-}, loss of this plasmid had no effect on adhesion. Dytoc *et al*., (1993) have reported *in vivo* data that support the involvement of this plasmid in intestinal adherence after oral inoculation of adult rabbits. *Escherichia coli* K-12 strain HB 101 containing this plasmid adhered to rabbit intestinal cells whereas HB101 without the plasmid did not adhere. In both rabbit (Li *et al*., 1993) and gnotobiotic piglet (Tzipori *et al*., 1987) models of disease,

the presence or absence of this plasmid made no difference to the amount of diarrhoea or intestinal histopathology. On the other hand, Wadolkowski *et al*., (1990) showed that both O157: H7 strain 933, and its plasmid-cured derivative 933cu, could individually colonize the gut of streptomycin treated mice but that 933cu could not establish colonization when used together with 933.

The conflicting findings about the role of pO157 may be attributable to differences in growth and assay conditions, and to differences between O157: H7 strains and to the fact that the large plasmid itself appears to be heterogeneous even within the O157: H7 serotype (Barrett *et al*., 1992). In addition, a serious limitation to establishing a role for pO157 in pathogenesis is that there is no suitable animal model that reproduces all aspects of the EHEC disease, from intestinal inoculation to bloody diarrhoea to renal involvement. The degree to which any of these animal models mimics the colonization mechanisms in humans is uncertain (Paton and Paton, 1998).

Epidemiological evidence indicates a stronger correlation between the presence of this plasmid and the development of HUS, rather than diarrhoea. As described above, the enterohaemolytic phenotype encoded on this plasmid was observed in 16 of 18 (88%) O111: H^- strains isolated from patients with HUS and in only 4 of 18 (22.2%) O111: H^- strains from patients with diarrhoea without HUS (Schmidt and Karch, 1996).

Despite the uncertainty about the significance of plasmid pO157 in disease, it is in fact widely distributed among human EHEC isolates. The initial study by Levine (1987) on the distribution of this plasmid among human isolates found that 99% of 107 O157: H7 strains possessed the plasmid, as did 77% of 44 O26: H11 strains. pO157 was also found in 81% of 26 Stx-positive strains of serotypes other than O157: H7 and O26: H11 (Levine, 1987). A subsequent study with a different strain collection from Europe showed similar results, with the plasmid being present in 60% of Stx-positive strains of serotypes other than O157: H7 and O26: H11 (Willshaw *et al*., 1992). Another study in Germany found pO157 in 90% of all Stx-producing *E. coli* isolates from patients. In addition to the 94-to104-kb pO157 plasmid, a number of other plasmids ranging in size from 2 to 87 kb have been found in strains of *E. coli* O157: H7 (Willshaw *et al*., 1992). However, there has been no

correlation has been seen between the possession of any of these plasmids and clinical disease.

2.4.9 Locus of enterocyte effacement (LEE)

Some bacterial pathogens possess the ability to produce a characteristic histological lesion called an attaching and effacing (A/E) lesion. The A/E lesion is defined by the intimate attachment between the bacteria and the epithelial surface and this phenotype is marked by a loss or effacement of microvilli on the intestinal epithelial cells at the site of bacterial attachment (Kaper *et al*., 2004). Enterohaemorrhagic *E. coli* produce A/E lesion that is characterized by degeneration and effacement of intestinal epithelial cell microvilli, intimate adherence of the bacteria to the epithelial cells, and assembly of highly organized cytoskeleton structures in the cells beneath intimately attached bacteria (Law, 2000). One of the important characteristics of EHEC including *E. coli* O157: H7 is their ability to produce A/E lesions on a variety of cell types including enterocytes (Tzipori *et al*., 1987; Law, 2000).These lesions involve ultrastructural changes in the enterocytes including loss of enterocyte microvilli and intimate attachment of the bacterium to the cell surface. Other members of the A/E family include a large number of animal pathogens such as those that cause disease in rabbits (REPEC, RDEC-1), pigs (PEPEC), dogs (DEPEC), and mice (*Citrobacter rodentium*) (Goosney *et al*., 2000).

All genes necessary for A/E formation are encoded on a 35 kb chromosomal pathogenicity island known as locus of enterocyte effacement (LEE). It contains 41 predicted open reading frames (ORFs) in at least 10 operons (Elliott *et al*., 1999; Deng *et al*., 2001). Locus of enterocyte effacement can be divided into three general regions based upon the specialized functions that contribute to the A/E phenotype. The region on the left of LEE contains an *esc* gene for the TTSS apparatus which is responsible for the secretion of Esp (*E. coli* secreted proteins). Mutations in the *escV or escN* result in abolition of the secretion of the proteins, EspA, EspB, and EspD, which are involved in virulence of the organism (Jarvis *et al*., 1995). There are two genes in the center of LEE. The first gene is *eae*, which encodes a 94 kDa outer membrane adhesion intimin which is required for intimate adherence. The second

gene is *tir* which encodes 'translocated intimin receptor' (Tir). The third region of LEE contains at least three genes *espA, espB* (Donnenberg *et al*., 1997), and *espD* (Lai *et al*., 1997) that encode the secreted proteins, EspA (Kenny and Finlay, 1997), EspB (Donnenberg *et al*., 1997), and EspD (Lai *et al*., 1997) respectively, which are secreted by the TTSS pathway (Jarvis and Kaper, 1996). These proteins are essential for the bacterial mediated signal transduction events within the host cell including the tyrosine phosphorylation of Tir and A/E formation (McDaniel and Kaper, 1997). Locus of enterocyte effacement has 23 open reading frames (ORFs) of undefined function (Elliott *et al*., 1998; Elliott *et al*., 1999; McDaniel and Kaper, 1997). Elliot *et al*., (1998) reported that LEE also appears to encode for novel proteins involved in TTSS pathway, new secreted proteins, chaperons, and a regulator/repressor. All of these genes are conserved in EPEC, EHEC, and REPEC strains (Abe *et al*., 1997).

The transfer of LEE of EPEC is sufficient to confer the ability to from A/E lesion to non-pathogenic *E. coli* strains (McDaniel and Kaper, 1997), but that of EHEC is not sufficient (Elliott *et al*., 1999). Locus of enterocyte effacement of EHEC was unable to induce the formation of attaching and effacing (A/E) lesions or to stimulate the secretion of Esp when it was cloned in *E. coli* K-12. It is believed that EHEC requires other non-LEE encoded factors to produce cytoskeleton changes in host cells (Elliott *et al*., 1999). These factors have not been identified, but perhaps include regulators and other accessory factors (Goosney *et al*., 2000).

In general, LEE elements in EPEC and EHEC strains are 94% conserved at the amino acid level and differ by less than 2% in the regions encoding the protein translocation complex (Elliott *et al*., 1999). Locus of enterocyte effacement of *E. coli* O157: H7 also encodes a cryptic prophage at one end that is not present in the EPEC O127: H6 LEE. Perna *et al*., (1998) suggested that the prophage was inserted into the LEE after the island was already present on the chromosome and it is unlikely that this prophage codes for any known virulence function.

2.5 Clinical manifestations of EHEC infections

The clinical manifestations of EHEC O157 infection range from symptom-free carriage to non-bloody diarrhoea, HC, HUS, and death **(Figure 1.5)**. The average interval between exposure and illness is three days with incubation periods as short as one day and as long as eight days being reported. Most patients with HC recover spontaneously within seven days. Illness typically begins with abdominal cramps and non-bloody diarrhoea. Bowel movement may become bloody over the next couples of days with the amount of blood varying from a few small streaks to stools that are almost entirely blood. Over 70% of patients report bloody diarrhoea in the most serious cases (MacDonald *et al*., 1996; Ackers *et al*., 1998). Vomiting occurs in 30% to 60% of cases, and fever, usually low grade, can be documented in only 30%. The absence of fever may lead clinicians to favour non-infectious diagnoses such as intussusception, ischaemic colitis, haemorrhage, or inflammatory bowel disease. Abdominal tenderness may be pronounced, prompting surgery for a presumed appendicitis.

In most patients serum-leucocyte counts are usually elevated, but faecal leucocyte counts are less than ten for each high-powered magnified field even in the presence of bloody diarrhoea (Slutsker *et al*., 1997). Barium enema may demonstrate ‘thumb-printing’, suggestive of oedema and submucosal haemorrhage, especially in the region of the ascending and transverse colon. When endoscopy is carried out, colonic mucosa often appears oedematous and hyperaemic, sometimes with superficial ulcerations or pseudomembranes (Griffin, 1995). The percentage of cases that progress to HUS range from 3% to 7% in sporadic cases (Slutsker *et al*., 1997) to about 20% or more in some outbreaks (Carter *et al*., 1987). Haemolytic uraemic syndrome is typically diagnosed six days after the onset of diarrhoea (Griffin, 1995). Some patients have abnormal laboratory values, suggestive of an incomplete form of HUS. About 50% of patients with HUS need dialysis and 75% have erythrocyte transfusions. Acute neurological complications such as stroke, seizure, and coma develop in 25% of patients. Rare complications include pancreatitis, diabetes mellitus, and pleural and pericardial effusions.

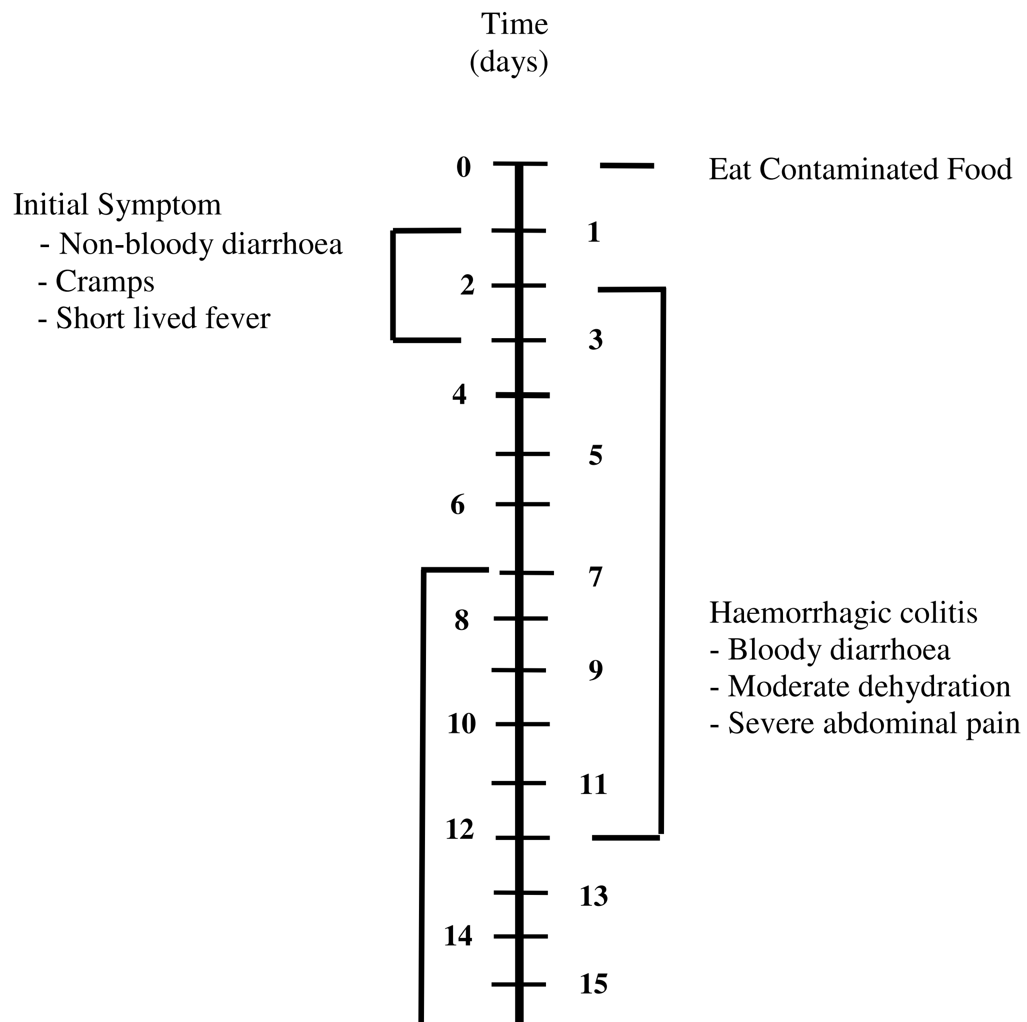


Figure 1.5 Symptoms and time course of enterohaemorrhagic *Escherichia coli* O157: H7 infection and its primary complications (from Tarr, 1995).

occasionally, patients with EHEC O157 infection are diagnosed as having thrombotic thrombocytopenic purpura, a condition similar to HUS but with more prominent neurological findings and less renal involvement. Among patients with HUS, about 3% to 5% die acutely and a similar percentage develop end-stage renal disease (Siegler, 1995). Many patients who regain renal function have chronic proteinuria, and some develop en-stage renal disease years or even decades later. Other long-term sequelae include cholelithiasis, colonic stricture, chronic pancreatitis, glucose intolerance, and cognitive impairment.

2.6 Complications

2.6.1 Haemolytic uraemic syndrome (HUS)

The term 'HUS' was introduced by Gasser *et al*., in 1954. It was earlier believed that *E. coli* serotype O111: B4 was the causative agent in the majority of the cases (Ruggenenti *et al*., 2001). However, it is now known that *E. coli* O157: H7 is the main causative agent of HC, out of which more severe infections can progress to HUS. About 2% to 7% of *E. coli* O157: H7 infections lead to this complication. Characteristic symptoms of HUS include pale skin, intravascular destruction of red blood cells, haemolytic anemia, depressed platelet count, decreased or no urine production, swelling, and acute renal failure (Meng *et al*., 2001; Ruggenenti *et al*., 2001). Several studies have revealed that children who have recovered from an acute episode of HUS are at risk for long-term complications including hypertension, renal insufficiency, end-state renal failure, and insulin-dependent diabetes mellitus (IDDM) (Scheiring *et al*., 2008). Renal failure is due to the production of Shiga toxins that damage endothelial cells which trigger the clotting mechanism. Within a week of infected persons developing symptoms for HC 15% may develop HUS which can lead to permanent loss of kidney function (Tarr *et al*., 2005).

Haemolytic uraemic syndrome occurs most commonly in children under 10 years of age. Argentina has a high incidence of HUS with 13.9 cases per 100,000 children younger than five years old reported in 2005 (Rivas *et al*., 2006a). In 5% to 10% of children in North America who are infected with *E. coli* O157: H7,

HUS develops soon after the onset of diarrhoea leading to the early development of chronic kidney failure (Wong *et al*., 2000). The mortality rate among children with HUS is 3% to 5% (Scheiring *et al*., 2008). Other suggested risk factors for the development of HUS include extremes of age, female gender, absence or weak presence of P1 antigen expression by red blood cells, bloody diarrhoea, fever, elevated leukocyte count, and treatment with an anti-mobility or antimicrobial agent (Siegler and Oakes, 2005). Various red blood cell antigens including P1 antigens have been proposed to either play a role in the development of Stx-mediated HUS or modulate the severity of resulting HUS (Jelacic *et al*., 2002).

2.6.2 Thrombotic thrombocytopenic purpura (TTP)

In 1924, Eli Moschcowitz described a 16-year-old girl who died within two weeks after the abrupt onset and progression of petechial bleeding, pallor fever, paralysis, haematuria, and coma. Disseminated microvascular thrombi were detected at autopsy, and these widespread thrombi in arterioles and capillaries, later found to be largely composed of platelets, remain the pathologic hallmark of Moschcowitz disease or TTP (Asada *et al*., 1985; Hosler *et al*., 2003). Clinical and laboratory feature still considered to be the key diagnostic criteria such as microangiopathic haemolytic anemia with fragmented erythocytes in the peripheral blood smear, thrombocytopenia, neurologic signs and symptoms, renal dysfunction, and fever. In routine clinical practice, TTP was often diagnosed in adult patients with predominant neurologic symptoms whereas a diagnosis of HUS was often made in children with predominant renal failure.

Numerous hypotheses on the etiology and pathogenesis of TTP have been put forward over the years. Among others, endothelial injury, the example by oxidative stress, decreased prostacyclin production, reduced fibrinolytic capacity of the vessel wall, anti-endothelial cell autoantibodies and specifically antibodies toward glycoprotein IV (CD36) (Tandon *et al.,* 1994; Schultz *et al*., 1998) that is located on microvascular endothelial cells and platelets, and the capacity of TTP plasma to induce apoptosis of microvascular endothelial cells (Laurence *et al*., 1996) have been proposed as pathogenetic factors. Moreover, a 37-kDa protein (Siddiqui *et al*., 1985),

and a 59-kDa protein or a calcium-dependent cysteine protease (Murphy *et al*., 1987; Kelton *et al*., 1996) were identified in serum from patients with acute TTP and suggested to be responsible for *in vivo* platelet aggregation. Moake *et al*., (1982) reported the presence of unusually large von Willebrand factor (ULVWF) multimers in plasma of four patients with a chronic relapsing course of TTP during remission. They suspected that these highly polymeric von Willebrand factor (VWF) multimers, similar in size to those found in endothelial cell culture supernatant, were responsible for *in vivo* platelet clumping in the microvasculature.

Plasma-refractory TTP patients or relapsing patients are often treated with more intensive plasma exchange regimens, the example twice daily. Splenectomy, best performed in remission after relapse, seemed to be often effective (Aqui *et al*., 2003), its beneficial effect may mainly relate to elimination of autoantibody-producing B cells (Kremer *et al.,* 2004), even though other mechanisms may underlie its beneficial effects. Recently, several patients with relapsing TTP caused by autoimmune-mediated. A disintegrin and metalloprotease with thrombospondin type 1 motifs (ADAMTS-13) deficiency have been treated with rituximab, a monoclonal anti-CD20 antibody, and successful short-term outcomes have been reported (Sallah *et al*., 2004). Whether there is any long-term advantage over corticosteroids, other immunosuppressive treatment or splenectomy, needs to be tested in prospective trails.

2.7 Laboratory diagnosis

Early diagnosis of EHEC infections in routine microbiological laboratories creates a basis for crucial acts such as treatment of the patient and prevention of additional cases of EHEC infection (Karch *et al*., 1999). The identification of strains of *E. coli* O157 worldwide is well-covered in routine microbiological laboratories with the use of differentiating media, especially Sorbitol-MacConkey agar (SMAC) (March and Ratnam, 1986; Paton and Paton, 2003), and commercial detection kits based on the agglutination and immunological reactions **(Table 1.3)**.

Table 1.3 Methods for the detection of enterohaemorrhagic *Escherichia coli*.

Diagnostic principle	Test method
Phenotypic methods	
1. Detection of *E. coli* O26, O91, O111, O103, O145, O157 antigens in bacterial culture	Latex agglutination, immunomagnetic separation (IMS)
2. Selective isolation of *E. coli* O157 bacteria based on colour reactions on agar plates	Chromogenic agars
3. Detection of enterohaemolysin production of bacterial culture on agar plate	Enterohaemolysin agar
4. Screening for Stx toxin or the O157 antigen in bacterial culture or stool	Enzyme immuno-assays (EIA)
5. Screening and separate detection of Stx1 and Stx2 toxins in bacterial culture or stool	Reverse passive latex agglutination (RPLA)
Genotypic methods	
1. Detection of genes encoding Stx, O specific regions or accessory virulence determinants in bacterial culture or stool	PCR, PCR combined with restriction fragment length polymorphism (PCR-RFLP) PCR combined with hybridization, colony hybridization, immunoblotting

data based on Karch *et al*., 1999; Perelle *et al*., 2004; Beutin *et al*., 2005; Prager *et al*., 2005.

Sorbitol MacConkey agar was developed by substituting the carbohydrate sorbitol for lactose in MacConkey agar, and SMAC agar has proven to be effective for the isolation of O157 STEC and is the most widely used medium for this purpose. The SMAC plate differentiates the non-sorbitol fermenting (NSF) isolates of serogroup O157 as colourless colonies compared to the other *E. coli* flora that typically ferment sorbitol (SF) and have a red phenotype. However, some enterobacterial genera or species, such as *Proteus, Providencia, Hafnia, Enterobacter,* and *E. hermanii*, also grow as colourless colonies and some of these species share common epitopes with the O157 antigen resulting in cross-reactions (March and Ratnam, 1986; Lior and Borczyk, 1987; Karch *et al*., 1999). In addition, *Yersinia enterocolitica* O: 9 cross-reacts with human anti-O157 sera (Chart *et al*., 1991). Sorbitol-MacConkey agar plate containing inhibitory components, such as cefiximide and tellurite (CT-SMAC) for other bacterial species has also been used for detection of strains of NSF O157 strains. However, this medium has been shown to be inhibitory against some strains of SF O157 (Karch and Bielaszewska, 2001), and thus its use is limited. A considerable number of strains within the O157 and non-O157 serogroups have been shown to be sorbitol-fermentative, thus complicating phenotypic detection of these pathogens on SMAC. Rapid agglutination or immunomagnetic separation (IMS) tests using the serological capture and detection of the most common serogroups of non-O157 strains such as O26, O91, O103, O111, O128, and O145 have been exploited for the detection of these pathogens.

Based on the vast phenotypic diversity of EHEC, the most effective detection method for finding all EHEC isolates is the detection of Stx production as the presence of the Stx toxin and its associated genes by definition indicates an EHEC isolate. Several methods have been developed for detection of the toxins. Of these methods enzyme-immunoassays (EIA-assays) can detect the presence of Stx1 and Stx2 in a bacterial culture or stool sample. Most EIA tests are based on the sandwich method, where immobilized monoclonal or polyclonal antibodies have been used as ligands for Stx molecules. The bound Stx toxin and antibody-enzyme-conjugate of the kit forms a visible colour reaction, thus indicating the presence of the Stx toxin in the sample. In addition, reversed passive latex agglutination (RPLA) tests detect Stx1 and Stx2 in bacterial cultures. During incubation, the presence of Stx(s) in the sample is

indicated by agglutination of the Stx toxin and the latex bound anti-Stx molecules in the walls of the sample well in a microtiter plate. However, other bacterial genera such as *Campylobacter, Citrobacter, Pseudomonas,* and *Edwardsiella,* have also been observed to produce Stx, which might produce a bias towards false positive reactions using Stx testing for detecting EHEC (Beutin *et al*., 1996; Kehl *et al*., 1997; Mackenzie *et al*., 1998). Thus the isolation of the Stx-producing strain is crucial to allow for further testing of its other typical EHEC characteristics to allow confirmation of the causative strain's species and pathogroup.

In specialized laboratories, molecular genetic methods like polymerase chain reaction (PCR) and hybridization methods have been of great value in detection of the *stx* genes of EHEC and the subsequent isolation of the EHEC strain. Currently, many of these methods have also become commercially available. Of these detection methods, the PCR method enables the testing of a bacterial culture or a direct patient sample, such as a stool sample, to search for the virulence genes. However, pure bacterial cultures are preferred because stool samples typically contains inhibitory substances for the PCR-reaction that lower the specificity and sensitivity of the test (Paton and Paton, 2003). Also, for the detection of EHEC, the time-lapse between the onset of EHEC infection and the sampling of the specimen will affect the rate of recovery and overall detection of EHEC isolates. Human faecal specimens collected two days after the onset of symptoms have had an isolation rate of 100% for *E. coli* O157: H7, but the rate can decrease to 33% for specimens collected seven days after infection (Thomson-Carter, 2001). For the recovery of EHEC O157 especially, enrichment procedures have been used successfully, in particular with the combination of the IMS technique (Karch *et al*., 1999; Thomson-Carter, 2001).

2.8 Drugs used in the treatment of EHEC infections

Diarrhoea associated HC is usually self-limiting, patients with HUS require early and careful management of acute renal failure with fluid and electrolyte balance. About 50% of the patients diagnosed with HUS require dialysis, and 75% need blood and platelet transfusions. In the adult form of HUS, fresh frozen plasma (FFP) is

recommended, especially if the patient has neurologic symptoms (Shah and Rand, 2003), most persons recover from HC without antibiotics or other specific treatment within five to ten days. There is no evidence that antibiotics improve the course of the disease and it is thought that treatment with some antibiotics may precipitate kidney complications. Antidiarrhoeal agents, such as Imodium® (Loperamide hydrochloride), should also be avoided. In children who are infected with *E. coli* O157: H7, treatment with antibiotics before the onset of diarrhoea may lead to a cessation of faecal shedding of the organism however, this does not prevent HUS (Wong *et al*., 2000). In the only published randomized prospective study, trimethoprim-sulfamethoxazole did not decrease the duration of symptoms, duration of pathogen excretion, or incidence of HUS. However, the study was small and therapy had not stared until a mean of seven days after the onset of illness (Proulx *et al*., 1992). A novel approach to preventing HUS involves the administration of a Stx-binding resin early in the infection with *E. coli* O157: H7. However, initial phase II trials have not shown a significant reduction in the rate of progression to HUS (Besser *et al*., 1999). Shiomi *et al*., (1999) indicated that oral fluoroquinolone therapy administered within three days of illness was effective in preventing the development of HUS.

The risk of HUS may be increased by antibiotic treatment as the antibiotics can result in the release of Shiga toxin from injured bacteria in the intestine, making the toxin more available for absorption (Wong *et al*., 2000). It is to be noted that *E. coli* strains are highly variable in their antibiotic-induced release of Shiga toxin (Wong *et al*., 2000). The antibiotics that show this effect include fluoroquinolones, cephalosporins, and trimethoprim-sulfamethoxazole (Wong *et al*., 2000). Indeed, *in vitro* data has demonstrated that ciprofloxacin or subinhibitory concentration of trimethoprim-sulfamethoxazole induced Stx production by *E. coli* O157: H7 (Walterspiel *et al*., 1992). Whether this occurs *in vivo* or has any clinical relevance is not yet known. However, in this study, patients were examined for faecal Stx, and no Stx was detected in the stools of patients treated with oral fluoroquinolones. In contrast Kimmitt *et al*., (1999) found that 4-quinolones rapidly induced an increase in the expression of the *stx* genes. Their findings indicated that 4-quinolones have the potential to induce massive toxinosis in individuals carrying substantial numbers of STEC. Available evidence indicates that the mechanism of

regulation is via the SOS response, the DNA repair mechanism that is stimulated by 4-quinolones (Drlica and Zhao, 1997).

2.9 Prevention and control

Prevention of infection requires control measures all at stages of the food chain, from agricultural production, manufacturing, and to the preparation of foods. Hazard analysis critical control point (HACCP) system can reduce the risk of *E. coli* O157: H7 infections. The control system for a manufacturing plant listed skinning, post-skinning rinsing, bactericidal spray, evisceration, final bactericidal rinse, chilling and maintenance of refrigeration. Farms, an important component of HACCP applications in animal production requires reduction of the carriage of *E. coli* O157: H7 by animals. Two approaches that have potential are competitive exclusion and vaccination. The slaughterhouse, is assumed to be the ultimate source of *E. coli* O157: H7 on carcasses by faecal contamination during animal production and slaughter operations. Traditional trimming procedures can reduce *E. coli* O157: H7 levels on areas of the carcass with visible faecal contamination (Hardin *et al*., 1995). Steam vacuum systems are used for spot removal and steam pasteurization cabinets are used for whole carcass treatments (Dorsa *et al*., 1996). Food processing *E. coli* O157: H7 can be controlled by traditional thermal processing techniques in pasteurization and D-values (decimal reduction time, the time required to destroy 90% of the population) which have been determined for a number of different temperatures in various ground meat and poultry products (Doyle and Schoeni, 1984; Ahmed *et al*., 1995). The D-values of *E. coli* O157: H7 have been reported in ground products and range from 0.4 to 0.8 min at 60 ^{o}C. Alternative technologies to thermal processing that control *E. coli* O157: H7 are the use of ionizing radiations for meat and poultry products with radiation pasteurization doses of 1.5 to 3.0 kGy appearing to be sufficient to eliminate *E. coli* at the levels that they are likely to occur in ground beef (Thayer and Boyd, 1993; Clavero *et al*., 1994). Home and food service undercooking has been an important contributing factor in *E. coli* O157: H7 outbreaks associated with ground beef. In particular, adequate cooking temperature and time, can prevent cross contamination

between raw and cooked foods and appropriate refrigerated storage are key factors for reducing the risks associated with *E. coli* O157: H7.

2.10 Medicinal plants

Medicinal plants are being increasingly studied by pharmacological researchers and many medicinal plants have a long history of medicinal use in Asia (Sinclair, 1998). These herbs have many potential clinical and therapeutic applications in the modern medical setting as numerous studies have demonstrated that they contain bioactive components and have resulted in a better understanding of their physiological, therapeutic, and clinical actions (Merken *et al*., 2001). Antimicrobial agents can also be derived from herbs, and over 1,000 plants exhibit antimicrobial effects (Grover *et al*., 2002). Traditionally, these herbs are said to provide safe and effective treatments against many diseases.

Many kinds of medicinal plants have been studied for their antibacterial activities against *E. coli* O157: H7 (Okubo *et al*., 1998; Takahashi *et al*., 1999; Cutter, 2000; Isogai *et al*., 2000; Voravuthikunchai *et al*., 2002; Voravuthikunchai *et al*., 2004; Voravuthikunchai *et al*., 2005c; Voravuthikunchai and Limsuwan, 2006). The botanical descriptions of the five plants including *Camellia sinensis, Peltophorum pterocarpum, Punica granatum*, *Quercus infectoria,* and *Uncaria gambir* are described in detail as follows.

2.11 Properties of medicinal plants

2.11.1 *Camellia sinensis* (L.) Kuntze

Family:	Theacease
Common name	Green tea, Black tea, Chinese tea
Name (Thai)	-

Botanical descriptions

It is an evergreen shrub or small tree that is usually trimmed to below two metres when cultivated for its leaves. It has a strong taproot. The flowers are yellow-white, 2.5 to 4 cm in diameter, with 7 to 8 petals **(Figure 1.6)**.

Figure 1.6 ***Camellia sinensis*** **(L.) Kuntze.**

Pharmacological and antibacterial activities

The leaves have been used in traditional chinese medicine (TCM), and other medical systems to treat asthma, angina pectoris, peripheral vascular disease, and coronary artery disease. Leaf tea extracts have demonstrated antibacterial activity against pathogens such as *Streptococcus mutans*, *Staphylococcus aureus* and *E. coli* O157: H7 (Isogai *et al*., 2000).

Active phytochemicals

Important chemical constituents of leaves are catechin, epigallocatechin, epicatechin, epicatechin gallate, epigallocatechin gallate, and gallocatechin gallate.

2.11.2 *Peltophorum pterocarpum* (DC) Backer ex K. Heyne

Family:	Fabaceae
Common name	Copperpod, Copper pod tree, Golden flamboyant, Yellow flamboyant, Yellow flame tree, Yellow poinciana
Name (Thai)	Nontri

Botanical descriptions

Yellow poincianas are usually planted in tropical Asia and South Pacific as specimen trees or as shade trees **(Figure 1.7)**.

Figure 1.7 ***Peltophorum pterocarpum*** **(DC) Backer ex K. Heyne.**

Pharmacological and antibacterial activities

Significant antibacterial activity of this medicinal plant against two important pathogenic bacteria, MRSA (Voravuthikunchai and Kitpipit, 2003) and EHEC O157: H7 (Voravuthikunchai *et al*., 2002; Voravuthikunchai *et al*., 2004) have been reported from this laboratory.

Active phytochemicals

Important chemical constituents of its bark are berginin, hirsutidin, propelargonidin, quercetin-3-O-β-D-diglucoside, rhamnetin, and rhamnetin-3-O-β-D-glucoside.

2.11.3 *Punica granatum* Linn.

Family:	Punicaceae
Common name	Pomegranate, Punic apple, Granades, Grnats, Carthaginian apple
Name (Thai)	Tubtim

Botanical description

The pomegranate is a shrub, usually with multiple stems, that commonly grows 1.8 to 4.6 m tall. It is found in Asia and the Middle East to the Himalayas. The fruit is technically a berry **(Figure 1.8)**. It is filled with crunchy seeds each encased in a juicy, somewhat acidic pulp that is itself enclosed in a membranous skin.

Figure 1.8 ***Punica granatum* Linn.**

Ethnomedical uses

The flowers and rind of the fruit are astringent and have been used for arresting chronic mucous discharges, passive haemorrhages, disorders of the mouth, night sweats and diarrhoea.

Pharmacological and antibacterial activities

Many workers have demonstrated significant activity of *Punica granatum* against MRSA (Holetz *et al*., 2002; Machado *et al*., 2003; Braga *et al*., 2005; Voravuthikunchai and Kitpipit, 2005a). In contrast, there have been very few reports that indicate that it can be active against Gram-negative bacteria. However, a methanolic extract of its pericarp showed good activity against *Proteus vulgaris* (Prashanth *et al*., 2001). In addition to its antibacterial activity, the active fractions of the pericarp have been reported to inhibit Verocytotoxin production by *E. coli* O157: H7 (Voravuthikunchai *et al*., 2005b).

Active phytochemicals

Pomegranate rind contains about 20% of tannin, consisting of two astringent principles, gallotanic acid and punicotannic.

2.11.4 *Quercus infectoria* G. Olivier

Family:	Fagaceae
Common name	Nut Galls, Aleppo Galls, White gall, Galls, Oak Galls
Name (Thai)	Benganee

Botanical descriptions

An evergreen shrub growing to 1.8 m, valued for excrescences formed upon the young branches. They are the result of a puncture made in the bark by an insect, *Diplolepis gallse tinctoriae* or *Cynips quercufolii* for the purpose of depositing its egg. The excrescences vary from the size of a large pea to that of a small hickory-nut, are nearly round, hard, and quite smooth with the exception of small tubercles scattered over the surface **(Figure 1.9)**.

Figure 1.9 Nut galls of *Quercus infectoria* G. Olivier.

Ethnomedical uses

Any nut galls produced on the tree are strongly astringent and can be used in the treatment of haemorrhages, chronic diarrhoea and dysentery. They may be used as a wash and gargle in a case of sore throat and as an injection in bad leucorrhoea; in which cases they arrest putrefactive tendencies, and may be combined with suitable stimulants. By coagulating the blood, they frequently will arrest haemorrhage from small vessels and sometimes are used for bleeding piles, both as an ointment and suppository.

Pharmacological and antibacterial activities

The pharmacological effect of nut galls have been revealed using various extraction methods. Intraperitoneal injection of a crude methanolic extract of nut galls was demonstrated to be effective as an analgesic and a CNS depressant in rats and also showed hypoglycemic activity in a female rabbit but only weak antiparkinson activity in the mouse (Dar *et al*., 1976). A similar extract also inhibited alpha-glycosidase enzymes such as sucrase, maltase, and isomaltase. This effect was comparable to acarbose, which is known to be a hypoglycemic agent (Hwang *et al*., 2000). A dried acetone-methanolic extract of nut galls showed an analgesic activity in the rat and significantly reduced blood sugar levels in a rabbit. A subfraction of this extract prepared by chloroform-methanolic extraction also had CNS depressant activity (Dar *et al*., 1976). *In vitro*, methanolic and aqueous extract of nut galls also showed an inhibitory effect on molluscicidal (Redwane *et al*., 1998), hepatitis C virus protease (Hussein *et al*., 2000), larvicidal (Redwane *et al*., 2002), and *Naja kaouthia* venom (Pithayanukul *et al*., 2005).

Chemical consituents

The identification of chemical constituents from nut galls revealed that this plant contained a large amount of tannins, gallic acid, syringic acid, ellagic acid, β-sitosterol, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, methyl oleanate, and hexagalloyl glucose (Dar *et al*., 1976; Ikram and Nowshad, 1977; Hwang *et al*., 2000), coumarin, betulinic acid methyl ester, triterpene and steroids (Dar *et al*., 1976). It also contained 1,2,6,-tri-O-galloyl-*β*-D-glucose, 1,-2,-3,-6-tetra-O-galloyl-*β*-D-glucose, 1,-2,-3,-4,-6-penta-O-galloyl-*β*-D-glucose, 4-O-digalloyl-1-2-3-6-tetra-O-galloyl-*β*-D-glucose, 6-O-digalloyl-1,-2,-3,-4-tetra-O-galloyl-*β*-D-glucose, 6-O-digalloyl-1,-2,-3-tri-O-galloyl-*β*-D-glucose, 6-O-trigalloyl-1,-2,-3-tri-O-galloyl-*β*-D-glucose, and 2,6-bis-O-digalloyl-1,3-di-O-galloyl-*β*-D-glucose (Nishizawa *et al*., 1983).

2.11.5 *Uncaria gambir* (Hunter) Roxb.

Family:	Rubiaceae
Common name	Cat's claw
Name (Thai)	-

Botanical descriptions

It is a small creeping herb. The leaves are oval-shaped with tapered ends **(Figure 1.10)**.

Figure 1.10 *Uncaria gambir* (Hunter) Roxb.

Ethnomedical uses

Leaves and branches of this plant have been used to treat inflammation and enhance the immune system.

Pharmacological and antibacterial activities

Very limited information on its antibacterial property has been reported. Results from our laboratories indicate high activity of this medicinal plant

against two important pathogenic bacteria, MRSA (Voravuthikunchai and Kitpipit, 2003) and *E. coli* O157: H7 (Voravuthikunchai *et al*., 2002; Voravuthikunchai *et al*., 2004).

Active phytochemicals

Important chemical constituents are catechutannic acid 22% to 25%, pyrocatechol 30%, catechin 33%, gambir-fluorescein, catechu red, and quercetin. Pyrocatechin and tannin are thought to be the main active components.

OBJECTIVES

To further investigate nut galls in the following aspects

1. To isolate and identify active antibacterial compounds in the nut galls
2. To determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of active antibacterial compounds in the nut galls against enterohaemorrhagic *E. coli* O157: H7
3. To evaluate the antibacterial activities of the compounds in the nut galls on enterohaemorrhagic *E. coli* O157: H7
4. To study their cytotoxicity to Vero cells
5. To perform an *in vivo* study of the effects of active compounds in the nut galls by using light microscope (LM), and transmission electron microscope (TEM)

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Test bacterial strains

Enterohaemorrhagic *E. coli* (EHEC) strains representing a variety of serotypes commonly implicated in major food-borne outbreaks were selected for testing. Two strains of *E. coli* O157: H7 (RIMD 05091078, RIMD 05091083) were isolated in a 1996 outbreak in Japan. RIMD 05091078 produced both VT1 and VT2, RIMD 05091083 produced only VT2. Another strain, EDL 933 isolated in an 1983 outbreak in USA produced both VT1 and VT2. Other VT producing strains *E. coli* O111: NM RIMD 05091056 (VT1), *E. coli* O26: H11 RIMD 05091055 (VT1), and *E. coli* O22 RIMD 05091556 (VT2) were also included. *Escherichia coli* ATCC 25922 was used as a reference strain. An overview of the isolated sources is given in **Table 2.1**.

2. Medicinal plant

Nut galls were purchased from medicinal plant stores. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmaceutical Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The plant materials were washed with distilled water, cut into small pieces, and dried at 60° C. They were then crushed in a mechanical mortar and extracted by ethyl acetate, n-butanol, acetone, ethyl acetate fraction, 95% ethanol, 50% ethanol, and water. Each extract was evaporated under reduced pressure in a rotary evaporator and stored in a sterile screw-capped bottle at -20 ^{o}C. For bioassay, all extracts were dissolved in 10% dimethylsulfoxide (DMSO, Merck, Germany) before use.

Table 2.1 Strains, Verocytotoxin, origin, and other features of the isolates.

Strains	Serotype	VT	Origin	Other features *
E. coli RIMD 05091078	O157: H7	VT1+VT2	human	Not examined
E. coli RIMD 05091083	O157: H7	VT2	human	Not examined
E. coli EDL 933	O157: H7	VT1+VT2	human	*espP, hlyA, eaeA*
E. coli RIMD 05091055	O26: H11	VT1	human	*espP, hlyA, eaeA*
E. coli RIMD 05091056	O111: NM	VT1	human	*espP, hlyA, eaeA*
E. coli RIMD 05091556	O22	VT2	bovine	(*eaeA*-)
E. coli ATCC 25922				

* = identified by PCR amplification

3. Animals

Female IRC mice, weighing between 25-30 g, age 9-10 weeks were provided by the Animal house, Faculty of Science, Prince of Songkla University, Thailand. They were housed under normal laboratory conditions at 25± 1 ^{o}C with a controlled 12 h light–dark cycle and maintained on standard rodent chow and tap water *ad libitum*. All animals received human care in compliance with the guidelines of the Animal Care and Use Committee of Prince of Songkla University, Thailand. When necessary, animals were deprived of food for 24 h with access to water *ad libitum* before the experiments.

4. Chemicals and media

Table 2.2 Chemicals and media.

Chemicals	**Company**
Acetone	Merck
Chloroform	Merck
Cell Counting kit	Wako
Dimethyl sulfoxide (DMSO)	Merck
Eosin Y	Electron Microscope Science
Ethanol (EtOH)	Merck
Ethyl acetate (EtOAc)	Merck
Ellagic acid	Fluka
EMbed-812 kit	Electron Microscope Science
E. coli O157 LPS detection kit	Nitto Denko
Fetal calf serum (FCS)	Sigma
Formalin solution (10%)	Electron Microscope Science
Gallic acid	Fluka
Hematoxylin	Electron Microscope Science
Lead citrate	Electron Microscope Science
Methanol (MeOH)	Merck
Mueller-Hinton agar (MHA)	Difco
Mueller-Hinton broth (MHB)	Difco
MacConkey agar	Difco
Eagle's minimal essential medium	Sigma
N-butanol	Merck
Osmium tetroxide (OsO_4), crystal	Electron Microscope Science
Paraformaldehyde, PRILLS, EM Grade	Electron Microscope Science
Propylene oxide, EM Grade	Electron Microscope Science
Paraffin embedding wax	Electron Microscope Science

Table 2.2 Chemicals and media (continued).

Chemicals	**Company**
Reversed passive latex agglutination (RPLA) test kit (*E. coli* Vero toxin detection kit)	Denka Seiken
Sorensen's phosphate buffer	Electron Microscope Science
Syringic acid	Fluka
Sorbitol-MacConkey agar (SMAC)	Difco
Standard antibiotics discs:	Oxoid
Amikacin (30 µg)	
Amplicillin (10 µg)	
Choramphenicol (30 µg)	
Erythromycin (10 µg)	
Kanamycin (10 µg)	
Norfloxacin (10 µg)	
Tetracycline (30 µg)	
Tannic acid (TA)	Fluka
Toluene	Merck
Toluidine blue O	Electron Microscope Science
Trypticase soy agar (TSA)	Difco
Trypticase soy broth (TSB)	Difco
Uranyl acetate	Electron Microscope Science
Xylene substitute	Merck

5. Equipment

Table 2.3 Equipment

Equipment	Model/Company
Analytical balance 5 digits	BP210S, Sartorius
Autoclave	SS-325, TOMY
Automatic tissues processor	Pathcenture, SHANDON
Diamond knife	DRUKKER
ELISA Reader	ELX 808 U, Bio-TEK
Embbeding system	EG1160, LEICA
Freeze dryer	Flexi Dry
Glass-knife maker	LKB 7800
Incubator	B5100E, Heraeus
Ion sputter	JFC-110, JAPAN
Laminar air flow cabinet	Micro flow
Light microscope	BH-2, Olympus
Micro-centrifuge	000 series, LABQUIP
Membrane filters nitrocellulose (0.22 and 0.45 µm)	Millipore
Micro-titerplate 96 wells	3599 Costar
Mass Spectrometer (MS)	JMS automass20, JEOL
Microtome	RM2235 LEICA
Nuclear magnetic resonance spectrometer Unity Inova 500 MHZ	VARIAN
Plate heater	MS-HP-3000
pH meter	Thermo,Orion
Rotavapor	R-200, BUCHI
Scanning electron microscope	JSM -5800 LV, JEOL
Semi-automatic critical point drying apparatus	Samdri-790
Transmission electron microscope	JEM-100CX II, JEOL

Table 2.3 Equipment (continued)

Equipment	**Model/Company**
Tissue floatation bath	MS 8513B Electrothermal
Ultramicrotome	MTXL, RMC
UV-Visible spectrophotometer	UV-1601, SHIMADZU
Vortex	G-560E, Scientific industry
Water bath	Julabo TW20

Methods

1. Preparation of ethanolic extract

An ethanolic extract was prepared by immersing 100 g of the dried nut galls material at least three times in 500 ml of 50% ethanol at 60° C for 30 min (Pithayanukul *et al.*, 2005) and then extracted in 500 ml 95% ethanol at room temperature for 3 days. The filtrate was evaporated to dryness under vacuum at 40° C using a vacuum rotary evaporator and the extracts again were evaporated to dryness at 40° C using a water bath. The successful extracted quantities were 51.70 g by 50% ethanol, and 34.50 g in 95% ethanol. The extract was tested for the presence of tannins by reacting it with a 5% ferric chloride test solution. Identification of the tannin types within the extracts were based on the development of blue-black and greenish colours for hydrolyzable tannins and a combination of condensed and hydrolyzable tannins, respectively (Evans, 1996).

2. Preparation of ethyl acetate, n-butanol, and acetone extracts

A dried nut gall sample (100 g) was percolated with 500 ml of each of the solvents listed below for 3 days. The filtrate was evaporated to dryness under vacuum at 40° C using a vacuum rotary evaporator and the extracts again evaporated to dryness at 40° C using a water bath. The extracted quantities for each solvent are as follows: ethyl acetate extracted 8 g, n-butanol 20 g, and acetone 18.19 g (Basri and Fan, 2005).

3. Preparation of ethyl acetate fraction

A dried nut galls sample (100 g) was percolated with 500 ml of acetone for 3 days. The filtrate was evaporated to dryness under vacuum at 40 ^{o}C using a vacuum rotary evaporator and the extracts again evaporated to dryness at 40° C using a water bath. The acetone extract was further fractionated with ethyl acetate using solvent by solvent method. The fraction was evaporated to dryness under

vacuum at 40° C using a vacuum rotary evaporator and the fraction again evaporated to dryness at 40° C using a water bath. The ethyl acetate fractionated was 13.47 g (Nishizawa and Nonaka, 1983).

4. Preparation of aqueous extract

A sample of dried nut galls (100 g) was macerated in 500 ml boiling water for 30 min and the mixture was then filtered. After cooling, the filtrate was freeze-dried to give a quantity of 54.58 g (Laupattarakasem *et al.*, 2003).

5. Phytochemical screening for the presence of active compounds

Phytochemical testing for the presence of active compounds such as flavonoids (Shinoda test), sterols and triterpenes (Lieberman-Burchard's test), and tannin and phenolic compounds (5% ferric chloride) was carried out. Biologically active phytochemicals constituents are shown in **Table 2.4**.

Table 2.4 Phytochemical constituents of 50% ethanolic extract and semi-purified Qi 4 fraction.

Parameter	50% ethanolic extract	semi-purified Qi 4 fraction
Appearance	slightly brown	dark brown
Test for tannin polyphenol		
5% ferric chloride reagent	+ + +	+ + +
Test for alkaloid		
Dragendoff's reagent	+ +	+ +
Mayer's reagent	+	+ + +
Test for cardiac glycoside	-	-
Kedde's reagent		
2% Potassium hydroxide in ethanol		
Test for sterols		
Liebermann-Burchard's test	+	+
Test for flavonoid		
Shinoda test	+	+

- = absent, + = slightly positive, + + = positive, + + + = strongly positive

6. Fractionation of the ethanolic extract of nut galls

The 50% ethanolic extract (41.158 g) was first subjected to separation by quick column chromatography using silica gel 60 GF_{254} (Merck) and then eluted stepwise with chloroform, methanol, and a linear gradient of chloroform:methanol (100:0 to 0:100 v/v). Consecutive 250 ml volumes of eluant were collected in flasks. An aliquot of the quick column fractions was spotted onto a thin layer chromatography (TLC) plate. The plates were developed with different proportions of chloroform: methanol: H_2O (6.0:3.7:0.3 v/v) as the mobile phase. After air-drying, the spots on the plate were located by exposure to a UV lamp. The collected volumes flasks were divided into five fractions as follows: fraction Qi 0 (flasks 1-9), fraction Qi 1 (flasks 10-11), Qi 2 (flasks 12-17), Qi 3 (flasks 18-26), and Qi 4 (flasks 27-100). There were then further separated on a silica TLC plate (TLC, Merck, 60 GF_{254}). The fractions were concentrated by vacuum evaporation and then each fraction was assayed for antibacterial activity.

7. Antibacterial assays

7.1 Paper disc agar diffusion method CLSI, 2006

A sterile filter paper disc (6 mm) was soaked with 10 μl of plant extract (250 mg/ml extraction solvent) so each disc was impregnated with 2.5 mg of the substance when its antimicrobial activity was to be examined. Bacterial suspensions were adjusted to a McFarland turbidity of 3.0 (approximately 9.0×10^8 cfu) and inoculated onto a Mueller Hinton agar (MHA, Difco, France) plate by dipping a sterile swab into the suspension and swabbing over the entire plate surface in three directions. Dry discs (dried at 37 ^{o}C overnight) with plant extract were applied to the surface of the MHA plate seeded with the test bacteria culture and then the cultures were incubated aerobically at 37 ^{o}C for 25 h. Antibiotic susceptibility discs (Oxoid) including amikacin (30 μg), amplicillin (10 μg), erythromycin (10 μg), chloramphenical (30 μg), kanamycin (10 μg), tetracycline (30 μg), and norfloxacin (10 μg) were used as controls. The antibacterial activity was evaluated by measuring

the annular radius of the inhibition zone. The experiments were performed in triplicate and the mean of the diameter of the inhibition zones (annular radii) was calculated.

7.2 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) by broth microdilution method

The minimal inhibitory concentrations (MICs) of ethanolic extracts of the nut galls were determined according to the Clinical and Laboratory Standards Institute (CLSI, 2006) method. Samples of 0.25 g weight was dissolved in 1 ml of 10% DMSO (250 mg/ml). Twenty µl of an overnight culture of each bacterial test strain containing approximately $5x10^5$ cfu was added to 160 µl of Mueller Hinton broth (MHB, Difco, France) supplemented with 20 µl of the extracts at final concentrations that ranged from 25 to 0.012 mg/ml. The tubes were incubated at 35° C for 18 h. Tests were performed at least in duplicate and the results were expressed as the lowest concentration of the extracts that produced complete suppression of colony growth, (MIC). The MBC was performed on the extracts that gave significantly low MIC values against each bacterial strain by subculturing those giving no growth in the MIC test onto fresh MHA. The concentration at which there was no visible bacteria growth after 24 h was regarded as the MBC. For the semi-purified Qi 4 fraction, 0.01 g extract was dissolved in 1 ml of 10% DMSO (10 mg/ml) and tested as above except that the 20 µl of the added extracts had final concentrations that ranged from 1000 to 0.48 µg/ml.

7.3 Time-kill assay

The bactericidal activity of the ethanolic extract and semi-purified Qi 4 fraction were studied using a time-kill assay. One hundred µl of a bacterial culture ($5x10^5$ cfu) was added to 800 µl of MHB containing 100 µl of plant extract and fraction at MIC, 2MIC, and 4MIC and incubated at 35 °C with shaking. Samples were collected at 2 h intervals until 24 h, and a control tube with 1% DMSO was used

as the control. Surviving bacteria were cultured on TSA and incubated under the same conditions for 24 h. All assays were carried out in duplicate.

8. Detection of Verocytotoxin production by *E. coli* O157: H7

8.1 Reversed passive latex agglutination (RPLA) test

Fraction Qi 4 (2MIC, 500 µg/ml) was diluted by 2-fold dilutions at concentrations that ranged from 2MIC, MIC, 0.5MIC, 0.25MIC, 0.12MIC, 0.06MIC, and 0.03MIC. Ten µls of a *E. coli* O157: H7 culture (grown in a Trypticase soy broth (TSB) at 37° C for 18 h) was added to each of the test tubes and incubated at 37 ^{o}C for 20 h. The culture was centrifuged at 5000 rpm for 5 min to separate the supernatant and cell pellets. Verocytotoxin from the periplasmic space was obtained by treating the cell pellet with 100 µl polymyxin B (5000 IU/ml) at 37 ^{o}C for 30 min (Karmali *et al.*, 1985). Verocytotoxin in the culture supernatant (mainly VT2) and cell-associated VT (periplasmic, VT1) were separately determined using a reversed passive latex agglutination (RPLA) test kit (*E. coli* Vero toxin detection kit; Denka Seiken Co; Tokyo), which can differentiate VT1 and VT2 using anti-VT1 antibody sensitized latex and anti-VT2 antibody sensitized latex.

8.2 Verotoxicity test

Culture supernatants and periplasmic extracts were filtered through membrane filters with 0.22 µm pores. These filtrates were tested for cytotoxic activity in a Vero cell assay system. Vero cells were maintained in Eagle's minimal essential medium (MEM) with 0.5% fetal calf serum (FCS) and gentamicin (100µg/ml) and grown as monolayers in 96-well microtiter tissue culture trays. Serial 10-or 2-fold dilutions of the test filtrates (10 µl) were added to wells containing 200 µl of MEM medium with 0.5% FCS. The microtiter trays were incubated at 37 ˚C in a 5% CO_2 atmosphere for 2 days, and the cell survival was examined by a colorimetric (MTT) assay (Cell Counting Kit; Wako Chemicals, Osaka). This assay detected living cells but not the dead cells. Sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-

nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (wst-1) was used to develop a quantitative colorimetric value for cell survival (Ishiyama *et al.*, 1993; Iwaki *et al.*, 1995). The reaction solution (10 µl) was added to all 96 wells, and the cells were incubated for an additional 4 h. The plates were read on a microplate reader (ELISA Reader ELX 808 U Bio-TEK Instruments, Incorporation, (U.S.A.), at a wavelength of 595 nm.

9. Hydrophobicity of the cell surface modified from Nostro *et al.*, 2004

Cell surface hydrophobicity of EHEC strains that had been grown in the presence and absence of nut galls extracts was assessed using the modified microbial adhesion test to hydrocarbon (MATH) assay as described by (Rozenberg *et al.*, 1980). In brief, 100 µl of culture was added to 800 µl sterile saline (0.85%) with 100 µl of the test extracts and semi-purified Qi 4 fraction (MIC, 2MIC, and 4MIC). The tubes were incubated at 37° C for 18 h. They were harvested by centrifugation at 3000 rpm for 10 min, washed twice, and resuspended in sterile saline so that the optical density (OD) at 600 nm was 0.3. The cell suspension was placed in a glass test tube and vortexed for 1 min with 0.25 ml of toluene (AR Grade). The tubes were agitated uniformly in a vortex mixer for 2 min and allowed to equilibrate at room temperature for 10 min. The toluene phase was separated from the aqueous phase and the OD of the aqueous phase was determined in disposable cuvettes before mixing with toluene and after phase separation. The OD of the aqueous phase was determined spectrophotometrically at 600 nm. Controls consisted of cells incubated with 1% DMSO. The hydrophobicity index (HPBI) was calculated as: (O.D.initial-O.D. final/O.D. initial) ×100%. *Escherichia coli* strains with a hydrophobic index greater than 70% was arbitrarily classified as hydrophobic (mordified from Nostro *et al.*, 2004).

10. *In vitro* studies on cell morphological changes after treatment with ethanolic extract and semi-purified Qi 4 fraction

Morphological changes induced by the MIC, 2MIC, and 4MIC of 50% ethanolic extract and semi-purified Qi 4 fraction were observed. Enterohaemoharrgic *E. coli* O157: H7 and reference strain were grown in Trypticase soy broth (TSB, Difco, France) at 37 ^{o}C for 6 h. An aliquot of the cell suspension (10^5 cfu) was added to broth containing the MIC concentration (0.78 mg/ml), 2MIC (1.56 mg/ml), 4MIC (3.12 mg/ml) of extract, and semi-purified Qi 4 fraction (250 µg/ml). The culture was incubated at 37 ^{o}C to determine cell morphological changes at 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, and 18 h using a transmission electron microscope and scanning electron microscope.

10.1 Scanning electron microscope (SEM)

The samples were centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 100 µl of 0.1 M phosphate buffer and then placed on a round microscope coverslip coated with poly-L-lysine and dried. The coverslip was then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 at 4 ^{o}C for 60 min. Specimens were washed 3 times with phosphate buffer for 15 min each time. After washing, they were placed in 1% osmium tetroxide solution for 1 h. Then they were dehydrated by ethanol at concentrations of 35, 50, 75, 90, and 100% for 15 min at each concentration. They were finally dried in a critical point dryer. Dried specimens were then fixed on the stub and coated with gold under vacuum by a sputter unit (10 nm thick) before SEM (JEOL JSM 5800 LV) examination at 15 kV acceleration voltages.

10.2 Transmission electron microscope (TEM)

The samples were centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 100 µl of 0.1 M phosphate buffer and were further fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 at 4 ^{o}C for 60 min. Specimens were

washed 3 times with phosphate buffer for 15 min each time. After washing they were placed in 1% osmium tetroxide solution for 1 h. There were then dehydrated by ethanol at the concentrations of 70, 80, 90, and 100%, infiltrated and embedded in Epon-812 resin (Electron Microscopy Sciences). One-micrometer-thick sections were stained with 1% toluidine blue and viewed with a light microscope. Ultrathin sections showing silver-grey interference were collected on copper grids and stained with uranyl acetate and lead citrate before viewing and photographing under TEM (JEOL JEM-100 CX II) at an accelerating voltage of 80 kV.

11. *In vivo* study on the effect of semi-purified Qi 4 fraction on mice

All the animals used in this study were supplied from the Animal House, Faculty of Science, Prince of Songkla University. Female IRC mice, aged 9 to 10 weeks, weight between 25 to 30 g, were used to test the effect of the semi-purified Qi 4 fraction on the colonization of mice by EHEC strains and any subsequent histopathological changes. Mice were divided into three groups as follows: control group, infected group, and treatment group. Each in the control group was given 0.2 ml sterile PBS. Animals in the treatment group was inoculated with *E. coli* O157: H7 RIMD 05091078 and administered daily with 0.2 ml semi-purified Qi 4 fraction to give final concentration of 0.5, 2.5, and 1 mg/ml for ten days. Throughout the experiment all mice had access to pellets and drinking water *ad libitum*.

11.1 Streptomycin treatment of mice

Streptomycin sulfate in drinking water (5 g/L) was given to all animals three days prior to inoculation and continued throughout the experiment.

11.2 Inoculation of animals

Mice were divided into three groups as follows: control group, infected group, and treatment group. Each in the control group was given sterile 0.2 ml PBS. To neutralize gastric acidity, 0.2 ml of 5% sterile sodium carbonate was given

intragastrically through a device attached to a tuberculin syringe and passed into the stomach. This was followed by 0.2 ml ($2x10^9$ cfu) of the appropriate broth culture of *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933.

11.3 Determination of shed bacteria in faeces and gastrointestinal tract at 10 days

Freshly expressed faecal pellets (approximately 0.1 g) were placed in 1 ml of normal saline and mixed with a vortex mixer. Ten µl of 10-fold serial normal saline dilutions of the homogenate were plated directly onto SMAC agar and incubated aerobically at 37 ^{o}C for 18 to 24 h. The colourless colonies were confirmed by slide agglutination with O157 antiserum (Denka Seiken Co., Ltd., Tokyo). The detection limit was 100 cfu/g faeces. To examine the numbers of *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933 in mouse organs, the animals were killed by spinal dislocation ten days after inoculation. Approximately 0.5 g of small intestine, caecum, and colon were removed aseptically. The tissues were homogenized separately in 1 ml normal sterile saline diluent with an Ultra-Turrax homogenizer. Bacterial numbers in the homogenates were determined by the loop dilution method. Culture plates were incubated under the appropriate conditions. The detection limit was 100 cfu/g tissue.

11.4 Histopathological examination of organs

After mice were killed, a specific segment (1 cm) of small intestine, caecum, colon, and kidney was removed aseptically. Each segment was rinsed in sterile normal saline. Specimens were fixed in 10% buffered neutral formalin and processed by standard procedures. Sections of paraffin-embedded tissues were stained with haematoxylin and eosin, then examined by light microscopy. All histological section were coded to assess histopathology without bias.

11.5 Ultrastructural examination

A segment was placed into fixative and cut into small pieces at right angles to the midline. The specimens were fixed by immersion in 4% paraformaldehyde buffered with 0.1 M phosphate pH 7.3 at 4 ^{o}C overnight. Fixed specimens were post-fixed in 1% osmium tetroxide for 4 h. This process was 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% ethanol for 5 min, 70% ethanol for 10 min, and 95% ethanol for 10 min. The specimens were subsequently soaked in absolute ethanol, followed by propylene oxide for 15 min twice. Tissues were then infiltrated with epoxy resin (Epon-812) by means of the following protocol: a mixture of epoxy resin:propylene oxide of (1:1) for 30 min, a mixture of epoxy resin:propylene oxide of (3:1) for 30 min, pure epoxy resin for two changes of 30 min each. The specimens were embedded in beam capsule size 00 and cured at 80 ^{o}C overnight. Tissue blocks were trimmed with a razor blade and sectioned with a diamond knife on a RMC MT-XL ultramicrotome (USA). 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 (Reynolds, 1963), again washed gently with double distilled water and allowed to dry.

12. Statistical analysis

Statistical analyses were performed by SPSS 10.0 for Windows. Independent sample *t-tests* were used as the test of significance. Values were considered significantly different if $P<0.05$. These data were expressed as mean± the standard error.

CHAPTER 3

RESULTS

1. Extraction of nut galls

The extraction yields using different solvents are shown in **Table 3.1**. Yields from an aqueous extract and a 50% ethanolic extract were higher (54.58 and 51.7% w/w, respectively) than for a 95% ethanolic, ethyl acetate, acetone, n-butanol, and ethyl acetate fraction. The amounts in the 95% ethanolic, 50% ethanolic, and aqueous extract were determined by a ferric chloride test. We found that ferric chloride test of the plant extracts produced a blue-black colour and this indicated the presence of hydrolyzable tannins.

2. Determination of antibacterial activity of different extracts

The MIC and MBC values of extracts of nut galls using different solvents is illustrated in **Table 3.2**. All different extracts were tested against *E. coli* O157: H7. The 50% ethanolic extract was highly effective against *E. coli* O157: H7 with the best MIC and MBC values of 0.19 and 0.19 mg/ml, respectively. It was interesting to note that the MIC and MBC values of the 50% ethanolic extract against *E. coli* O157: H7 gave the same result (0.19 mg/ml). This means that the 50% ethanolic extract of nut galls, has bactericidal activity against *E. coli* O157: H7.

3. Fractionation of the ethanolic extract of nut galls

The 50% ethanolic extract (41.158 g) was first subjected to separation by quick column chromatography using silica gel 60 GF_{254} (Merck) then eluted stepwise with chloroform and methanol followed by a linear gradient of chloroform:methanol (100:0 to 0:100 v/v). Fractionation of the ethanolic extract of nut galls is summarized in **Figure 3.1**.

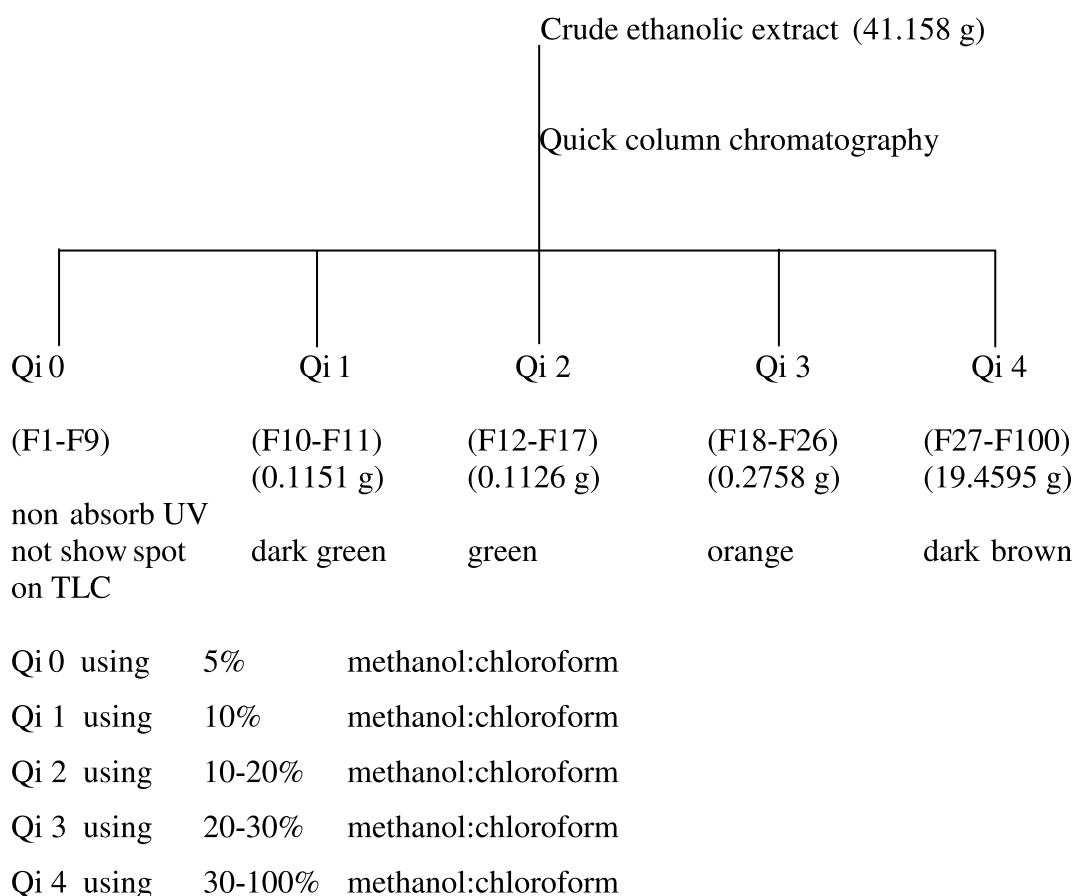
Table 3.1 Nut galls extraction by different solvents and its chemical constituents.

Crude extracts	Percent yield (w/w)	Chemical constituents
Ethyl acetate	8	ND
n-butanol	20	ND
Acetone	18.19	ND
Ethyl acetate fraction	13.47	ND
95% ethanolic	34.50	hydrolyzable tannins
50% ethanolic	51.70	hydrolyzable tannins
Aqueous	54.58	hydrolyzable tannins

ND = not determined.

Table 3.2 Minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) of extracts from nut galls using the broth microdilution method.

Extract	*E. coli* O157: H7 RIMD 05091078	
	MIC (mg/ml)	MBC (mg/ml)
Ethyl acetate	0.78	12.5
n-butanol	0.78	6.25
Acetone	0.78	1.56
Ethyl acetate fraction	0.39	3.13
95% ethanolic	0.39	0.78
50% ethanolic	0.19	0.19
Aqueous	0.78	0.78



Qi 0 using 5% methanol:chloroform

Qi 1 using 10% methanol:chloroform

Qi 2 using 10-20% methanol:chloroform

Qi 3 using 20-30% methanol:chloroform

Qi 4 using 30-100% methanol:chloroform

Figure 3.1 Fractionation of the ethanolic extract from nut galls by quick column chromatography.

4. Antibacterial assays

4.1 Paper disc agar diffusion method

The inhibition zones in the disk diffusion method using different solvents extracts and standard antibiotic discs are shown in **Table 3.3**. The products from the 95% ethanolic and 50% ethanolic extracts produced inhibition zones against all strains of EHEC and the reference strain. The inhibition zones ranged from 9 to16 mm. Both aqueous extracts of nut galls and tannic acid itself did not produce inhibition zones against the reference strain. All the isolated strains and the reference strain were susceptible to the three antimicrobial agents tested: amikacin, chloramphenicol, and norfoxacin while all the tested strains were resistant to erythromycin.

4.2 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) by broth microdilution method

Antibacterial activities of the 50% and 95% ethanolic extract against the six *E. coli* strains are shown in **Table 3.4**. Both the 50% and 95% ethanolic extract showed very strong activity against all *E. coli* with MIC and MBC values of 0.78 to 6.25, 1.56 to 12.5, and 0.19 to 0.78, 0.78 to 6.25 mg/ml, respectively. The significant antibacterial effects, expressed as MIC and MBC values of the Qi 2, Qi 3, and Qi 4 fractions against EHEC are shown in **Table 3.5**. The MIC and MBC values of Qi 2, Qi 3, and Qi 4 against all tested strains were in concentrations that ranged from 250 to 500 μg/ml. Due to its low yield (0.28%), fraction Qi 1 was only tested against *E. coli* O157: H7 RIMD 05091078 and *E. coli* ATCC 25922. It was demonstrated to have an MIC values too high (>1000 μg/ml) to be further considered. Antibacterial activities of tannic acid against EHEC are shown in **Table 3.6**. The MIC and MBC values of tannic acid against all tested strains were in the range of from 250 to 1000 μg/ml. The gallic acid, ellagic acid, and syringic acid were demonstrated to have very high MIC value (> 1000 μg/ml).

Table 3.3 Antibacterial activity of different extracts of nut galls (2.5 mg/disc) against *Escherichia coli* strains.

Extracts/Tannic acid/ Antibiotics	Mean values of inhibition zone (mm)						
	O157: H7 RIMD 05091078	O157: H7 RIMD 05091083	O157: H7 EDL 933	O26: H11 RIMD 05091055	O111: NM RIMD 05091056	O22 RIMD 05091556	*E. coli* ATCC 25922
Ethyl acetate	-	-	-	7.0	8.0	-	-
n-butanol	-	-	-	-	7.0	-	-
Acetone	-	-	-	-	7.0	-	-
Ethyl acetate fraction	-	-	-	-	8.0	-	-
95% ethanolic	13.0	13.0	11.0	16.0	13.0	13.0	11.0
50% ethanolic	11.0	10.0	9.0	14.0	12.0	11.0	10.0
Aqueous	12.0	12.0	10.0	12.0	12.0	10.0	-
Tannic acid	10.0	9.0	9.0	13.0	10.0	9.0	-
Amikacin 30 µg	21.6 S	22.5 S	23.2 S	24.7 S	25.8 S	25.4 S	26.8 S
Amplicillin 10 µg	20.0 S	21.8 S	21.2 S	22.7 S	-	18.5 S	19.5 S
Erythomycin 10 µg	11.3 R	13.5 R	11.7 R	10.2 R	13.7 R	18.7 I	-
Chloramphenicol 30 µg	24.1 S	26.7 S	26.8 S	25.6 S	29.7 S	24.7 S	27.7 S
Kanamycin 10 µg	23.3 S	22.0 S	22.7 S	23.0 S	-	24.6 S	25.5 S
Tetracycline 30 µg	24.7 S	-	26.7 S	27.6 S	8.7 R	26.8 S	28.7 S
Norfoxacin 10 µg	29.4 S	37.5 S	28.6 S	32.2 S	38.0 S	33.4 S	33.7 S

- = no zone, S = susceptible, I = intermediate susceptible, R = resistant.

Table 3.4 Antibacterial activity of nut galls by the broth microdilution method.

Strains	MIC/MBC (mg/ml)	
	50% ethanolic extract	95% ethanolic extract
E. coli O157: H7 (RIMD 05091078)	0.78/3.12	0.78/0.78
E. coli O157: H7 (RIMD 05091083)	1.56/3.12	0.78/0.78
E. coli O157: H7 (EDL 933)	1.56/1.56	0.78/0.78
E. coil O26: H11 (RIMD 05091055)	1.56/1.56	0.78/0.78
E. coli O111: NM (RIMD 05091056)	0.78/0.78	0.19/6.25
E. coli O 22 (RIMD 05091556)	1.56/12.5	0.78/0.78
E. coli (ATCC 25922)	0.39/6.25	0.78/3.12

Table 3.5 MICs and MBCs of Qi 1, Qi 2, Qi 3, and Qi 4 fractions against *Escherichia coli* strains using the broth microdilution method.

Strains	MIC/MBC (µg/ml)				
	Qi 1	Qi 2	Qi 3	Qi 4	Norfloxacin
E. coli O157: H7 (RIMD 05091078)	>1000/NA	500/500	500/500	250/250	0.12/0.12 (S)
E. coli O157: H7 (RIMD 05091083)	ND	250/250	500/500	250/500	0.12/0.12 (S)
E. coli O157: H7 (EDL 933)	ND	250/250	250/500	500/500	<0.06 (S)
E. coli O26: H11 (RIMD 05091055)	ND	500/500	500/500	250/250	0.12/0.12 (S)
E. coli O111: NM (RIMD 05091056)	ND	500/500	250/1000	500/500	<0.06 (S)
E. coli O22 (RIMD 05091556)	ND	250/250	250/500	500/500	<0.06 (S)
E. coli (ATCC 25922)	>1000/NA	250/250	250/500	500/500	<0.06 (S)

NA = not application, ND = not done, S = susceptible.

Table 3.6 MICs and MBCs of gallic acid, ellagic acid, tannic acid, and syringic acid against *Escherichia coli* strains by broth microdilution method.

Strains	MIC/MBC (µg/ml)			
	Gallic acid	Ellagic acid	Syringic acid	Tannic acid
E. coli O157: H7 (RIMD 05091078)	>1000	>1000	>1000	250/500
E. coli O157: H7 (RIMD 05091083)	>1000	>1000	>1000	500/1000
E. coli O157: H7 (EDL 933)	>1000	>1000	>1000	500/500
E. coli O26: H11 (RIMD 05091055)	>1000	>1000	>1000	500/1000
E. coli O111: NM (RIMD 05091056)	>1000	>1000	>1000	500/1000
E. coli O22 (RIMD 05091556)	>1000	>1000	>1000	1000/1000
E. coli (ATCC 25922)	>1000	>1000	>1000	>1000

5. Time-kill assay

5.1 The effect of 50% ethanolic extract

The effect of the 50% ethanolic extract against *E. coli* O157: H7 strains and the *E. coli* reference strain are shown in **Figure 3.2**. Figure 3.2A shows the time-kill study of *E. coli* O157: H7 05091078. At its MIC of 0.78 mg/ml, and 2MIC at 1.56 mg/ml growth was suppressed from about 6 h. At 4MIC (MBC) a killing effect was observed on *E. coli* O157: H7 within 12 h. Figure 3.2B shows the time-kill study of *E. coli* O157: H7 05091083. At its MIC of 1.56 mg/ml, a bacteriostatic effect was observed over the first 4 h followed by a slow lysis. At 2MIC (3.12 mg/ml) the killing effects was observed much earlier and all cells were dead within 10 h. Figure 3.2C shows the time-kill study of *E. coli* O157: H7 EDL 933. At its MIC of 1.56 mg/ml, both bacteriostatic and bactericidal effects were observed with all cells dead after approximately 20 h. Figure 3.2D shows the time-kill study of *E. coli* ATCC 25922. Bacterial growth occurred when treated with the extract at its MIC of 0.39 mg/ml, 2MIC (0.78 mg/ml), and 4MIC (1.56 mg/ml). At 8MIC (3.12 mg/ml), a bacteriostatic effect was observed over the 24 h period. Bactericidal activity was observed at a concentration of 16MIC (6.24 mg/ml) with all cells dead after 14 h.

5.2 Inhibitory effect of the semi-purified Qi 4 fraction

The effect of the semi-purified Qi 4 fraction against *E. coli* O157: H7 and the *E. coli* reference strain are shown in **Figure 3.3**. Figure 3.3A demonstrates the time-kill study of the O157: H7 05091078. At its MIC of 250 µg/ml, both bacteriostatic and bactericidal effects were observed. All cells were dead after 20 h. Figure 3.3B demonstrates the time-kill study of the O157: H7 05091083. At its MIC of 250 µg/ml, a bacteriostatic effect was produced after the first 4 h period but some recovery was observed after 18 h. At its 2MIC level of 500 µg/ml, a killing effect was observed on the bacterial cells and all cells were dead after approximately 16 h. Figure 3.3C and Figure 3.3D shows the time-kill studies of the O157: H7 EDL 933 and *E. coli* ATCC 25922. At its MIC of 500 µg/ml, both bacteriostatic and

bactericidal effects were observed. All cells were dead after about 18 h and 12 h respectively.

6. Detection of Verocytotoxin production by *E. coli* O157: H7

6.1 Reversed passive latex agglutination (RPLA) test

Evidence for the inhibition of the production of VT both in the periplasmic space (VT1) and cell supernatant (VT2) by fraction Qi 4 of nut galls is presented in **Table 3.7**. Since the highest yield of product, (79.37%) was fraction Qi 4 it was used for further experiments. *E. coli* O157: H7 RIMD 05091078 was cultured with fraction Qi 4 at 2MIC, MIC, and subinhibitory concentrations (0.5MIC, 0.25MIC, 0.12MIC, 0.06MIC, and 0.03MIC) for 20 h. The effect on VT production was detected immunologically. It was clear that fraction Qi 4 of nut galls markedly inhibited the production of VT both in the periplasmic space (VT1) and cell supernatant (VT2). At their highest concentrations (2MIC) to 0.12MIC of Qi 4, VT1 production was decreased by at least 64-fold, compared with the control values (without Qi 4). At 0.06MIC, VT1 level decreased by 16-fold. Similar patterns were obtained with extracellular VT2 production. At the lowest concentration (0.03MIC) of Qi 4, both VT1 and VT2 production was decreased 4-fold.

6.2 Verotoxicity test

The biological activity of the culture supernatant and polymyxin B extract in 20 h cultures with Qi 4 at different concentrations (0.03MIC to 2MIC) are shown in **Figure 3.4**. The extract from polymyxin B specimens treated with Qi 4 at 2MIC produced significantly less toxicity to Vero cells, compared to the control, ($P<0.05$). This correlated well with the results from the previous experiment. The 20 h culture supernatant treated with the fraction Qi 4 at 0.5MIC, MIC, and 2MIC produced less effect on Vero cells, compared to the control, ($P<0.05$).

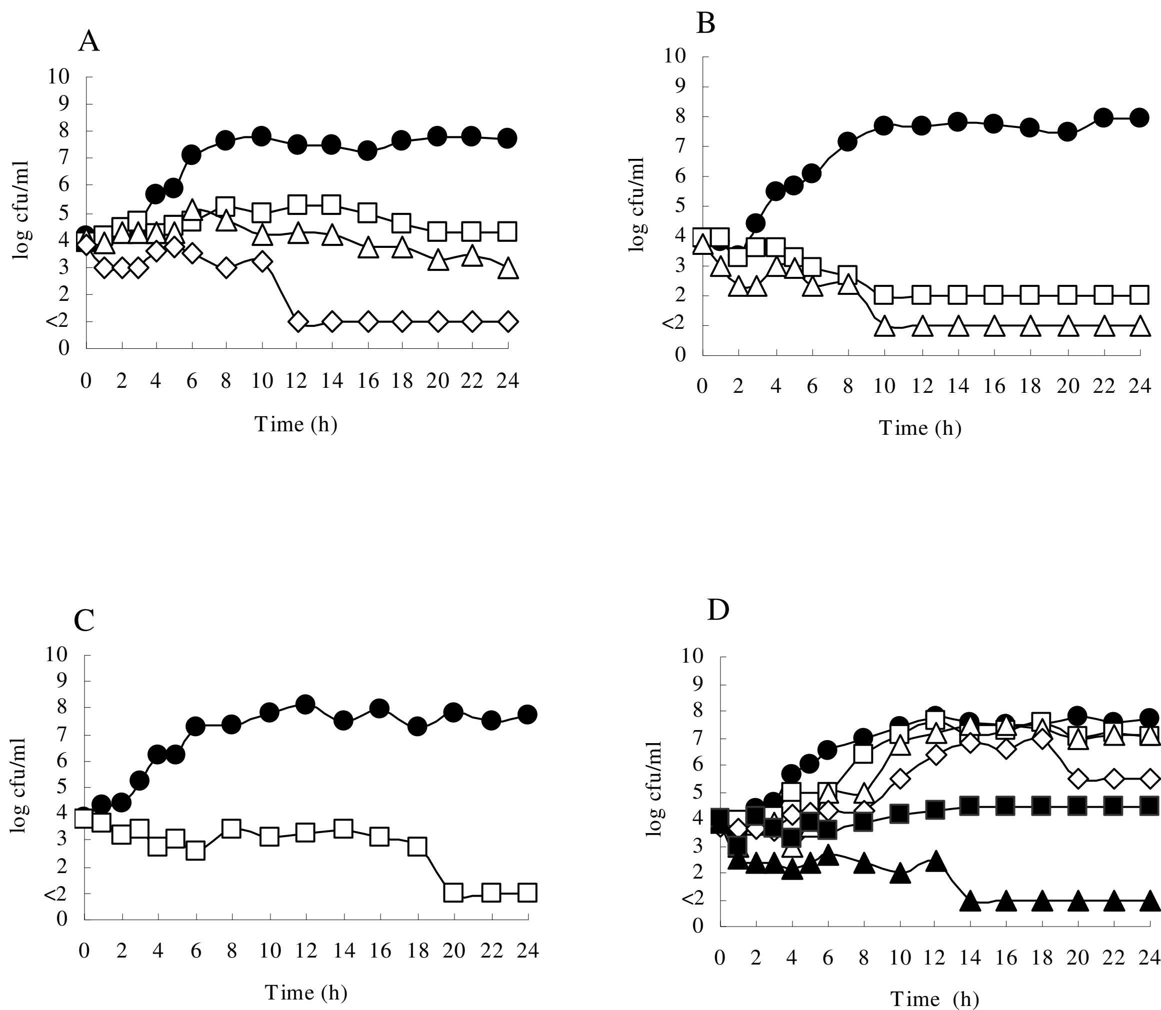


Figure 3.2 Growth curves of *Escherichia coli* in the presence of MIC and MBC levels of the 50% ethanolic extract at 37 ^{o}C for 24 h. (A) *Escherichia coli* O157: H7 RIMD 05091078; (□) MIC = 0.78 mg/ml, (△) 2MIC = 1.56 mg/ml, (◇) MBC = 3.12 mg/ml, (B) *Escherichia coli* O157: H7 RIMD 05091083; (□) MIC = 1.56 mg/ml, (△) MBC = 3.12 mg/ml, (C) *Escherichia coli* O157: H7 EDL 933; (□) MIC, MBC = 1.56 mg/ml, (D) *Escherichia coli* ATCC 25922; (□) MIC = 0.39 mg/ml, (△) 2MIC = 0.78 mg/ml, (◇) 4MIC = 1.56 mg/ml, (■) 8MIC = 3.12 mg/ml, (▲) MBC = 6.24 mg/ml, (●) control.

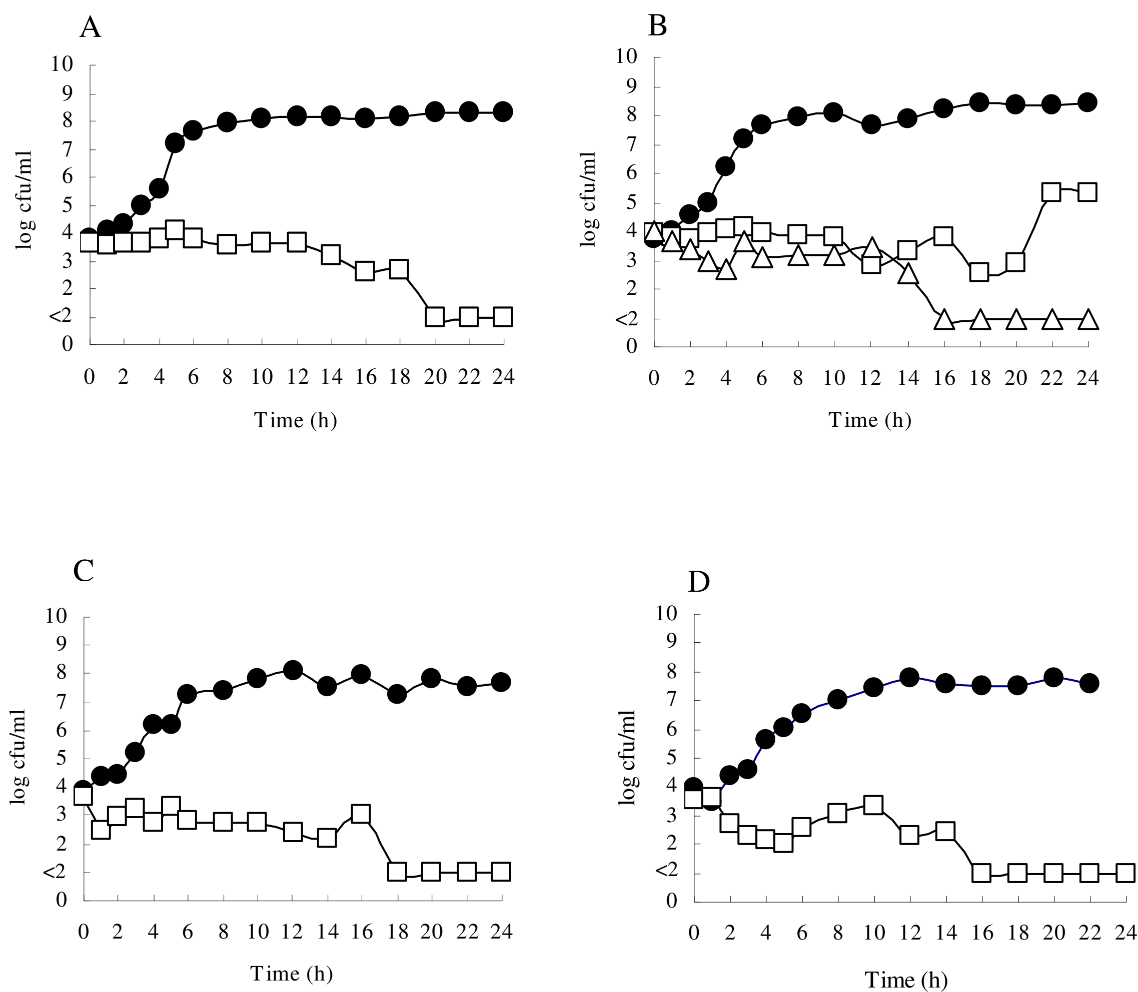


Figure 3.3 The growth curve of the *Escherichia coli* at 37 °C for 24 h in the presence of the MIC or MBC of the semi-purified Qi 4 fraction. (A) *Escherichia coli* O157: H7 RIMD 05091078; (☐) MIC, MBC = 250 µg/ml, (B) *Escherichia coli* O157: H7 RIMD 05091083; (☐) MIC, 250 µg/ml, (◇) MBC = 500 µg/ml, (C) *Escherichia coli* O157: H7 EDL 933; (☐) MIC, MBC = 500 µg/ml, (D) *Escherichia coli* ATCC 25922; (☐) MIC, MBC = 500 µg/ml, (●) control.

Table 3.7 Inhibitory effect of semi-purified fraction Qi 4 on Verocytotoxin production by *Escherichia coli* O157: H7 RIMD 05091078 at 20 h.

Concentrations of semi-purified fraction, Qi 4	VT titer	
	VT1 titer	VT2 titer
2 MIC	<2	<2
MIC	<2	<2
0.5 MIC	<2	<2
0.25 MIC	<2	4
0.12 MIC	2	4
0.06 MIC	8	32
0.03 MIC	32	32
E. coli O157: H7 RIMD 05091078	128	128

MIC of Qi 4 = 250 µg/ml.

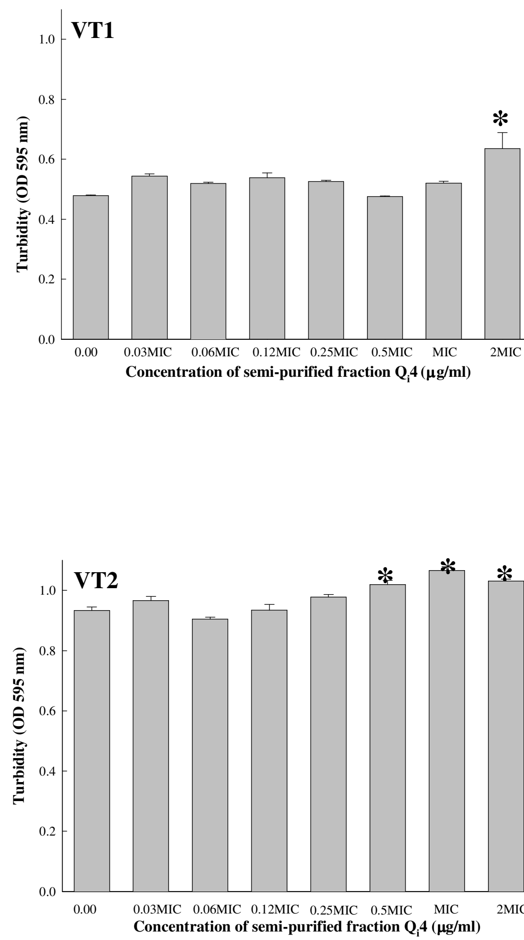


Figure 3.4 Effects of semi-purified fraction Qi 4 on Vero cells at 20 h. The data are represented as means ± the standard error, (*asterisks*) significantly higher than those of the controls ($P < 0.05$ in Dunnett test).

7. Hydrophobicity of the cell surface

The hydrophobicity index (HPBI) of the EHEC strains and *E. coli* ATCC 25922 are presented in **Figure 3.5**, **Figure 3.6**, and **Figure 3.7**. From **Figure 3.5** and **Figure 3.6**, the HPBI of the STEC strains were found to be far less than 70%, and this indicated the hydrophilic nature of these strains. The HPBI values of EHEC strains as well as *E. coli* ATCC 25922 **(Figure 3.7)** at sub-MICs of the extract did not show any definite patterns. In contrast, the growth of EHEC cells in the presence of 4MIC levels of nut galls extracts resulted in a change to moderate surface hydrophobicity (HPBI lower than 70%).

8. *In vitro* studies on cell morphological changes after treatment with ethanolic extract and semi-purified Qi 4 fraction

8.1 Treatment with 50% ethanolic extract at 4 h

The effects of the 50% ethanolic extract on *E. coli* O157: H7 RIMD 05091078 are shown in **Figure 3.8** and **Figure 3.9**. In Figure 3.8A, bacterial cells exhibited a normal smooth surface morphology, the normal length of bacterial cells was from 1.2 to 2.3 µm, with perithichous flagella present. In Figure 3.8B, at 4MIC (3.12 mg/ml) bacterial cells demonstrated 80% coccoid forms with a diameter range of 1 µm, smooth surface morphology, abnormal cell divisions, blebs on the cell wall surface in rod-shaped forms, and the disappearance of flagella. In Figure 3.9B, at 4MIC (3.12 mg/ml), TEM revealed blebs on the cell wall surface, the cell wall was irregular with undulations, and electron dense bodies appeared within the cytoplasm. At 12 h, the effects of the 50% ethanolic extract on *E. coli* O157: H7 are shown in **Figure 3.10** and **Figure 3.11**. Scanning electron microscope revealed untreated cells with rod shapes, peritrichous flagella, and a smooth surface. Morphological changes of bacterial cells treated with ethanolic extract showed bacterial cells of abnormal length and loss of flagella (Figure 3.10B and Figure 3.10C). Bacterial cells treated with the extract at 4MIC (3.12 mg/ml) showed collapsed cell walls. Transmission electron microscope of *E. coli* O157: H7 treated with 1% DMSO revealed that cells

had typical rod shapes, double layers of outer membrane closely apposed to the cytoplasmic membrane and dispersed nuclear material (Figure 3.11A). The bacterial cells treated with the extract at MIC (0.78 mg/ml) showed electron dense intracellular material. Transmission electron microscope micrographs of *E. coli* O157: H7 treated with the extract at 2MIC (1.56 mg/ml) demonstrated that disruption in the outer and cytoplasmic membranes at the polar regions of the cells, and some vacuolization were seen (Figure 3.11C). Bacterial cells treated with the extract at 4MIC (3.12 mg/ml), had vacuoles and electron dense material in the cytoplasm (Figure 3.11D).

8.2 Semi-purified Qi 4 fraction at 12 h

The effect of the semi-purified Qi 4 fraction on *E. coli* O157: H7 RIMD 05091078 and *E. coli* ATCC 25992 are shown in **Figure 3.12** and **Figure 3.13**. In Figure 3.12A, cells treated with 1% DMSO showed normal smooth surface morphology with a normal length of from 1.2 to 2.3 μm and peritrichous flagella. By comparison in Figure 3.12B, at MIC (250 μg/ml), the bacterial cells showed abnormal cell division, blebs on the cell wall surface, and loss of flagella. In Figure 3.13A, the cells treated with 1% DMSO revealed dispersed nucleoid material packed with ribosomes, and cell walls consisting of an electron dense outer membrane, an inner plasma membrane with an electron transparent periplasmic spaces in between the two membranes. Treatment of the bacterial cells with MIC (250 μg/ml) revealed separation of the plasma membrane from the outer membrane (Figure 3.13B). Similar changes in the plasma membrane of *E. coli* ATCC 25992 were also seen when it was treated with the semi-purified Qi 4 fraction at MIC (250 μg/ml) Figure 3.13C.

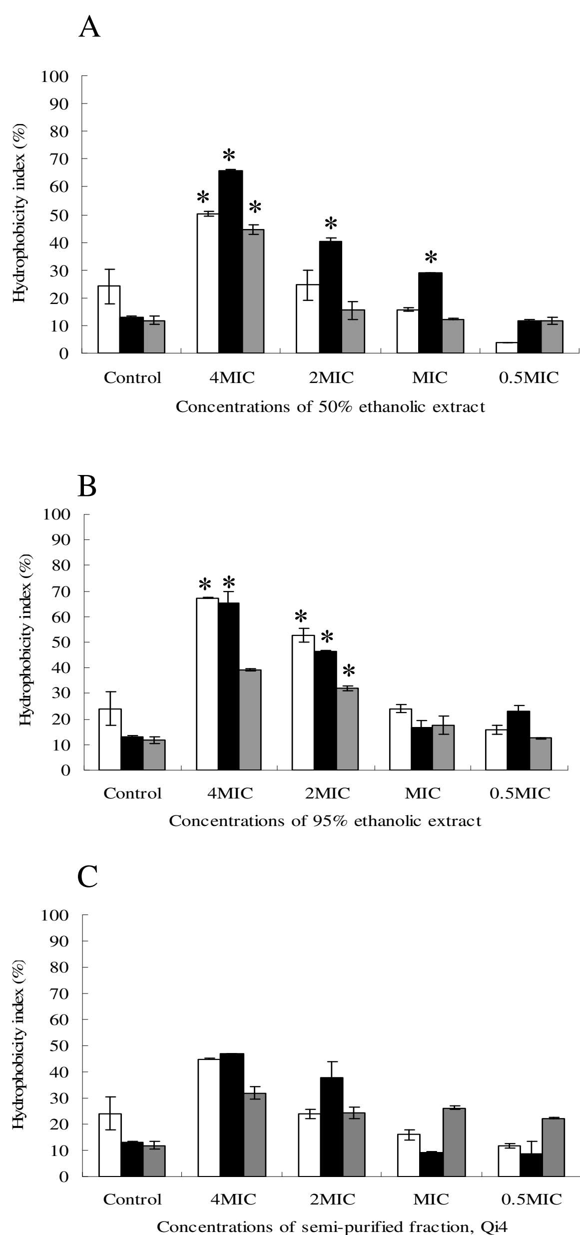


Figure 3.5 The mean hydrophobicity index at 18 h, as measured by the percentage adhesion to toluene, of *Escherichia coli* O157: H7 treated with (A) 50% ethanolic extract, MIC = 0.78, 1.56, and 1.56 mg/ml, (B) 95% ethanolic extract, MIC = 0.78, 0.78, and 0.78 mg/ml, (C) semi-purified fraction, Qi 4, MIC = 250, 250, and 500 µg/ml. (□ = RIMD 05091078; ■ = RIMD 05091083; ▩ = EDL 933, and control = 1% DMSO). The *asterisks* indicate $P<0.05$ vs. control, by ANOVA and Fisher's least-significant differences (LSD) test.

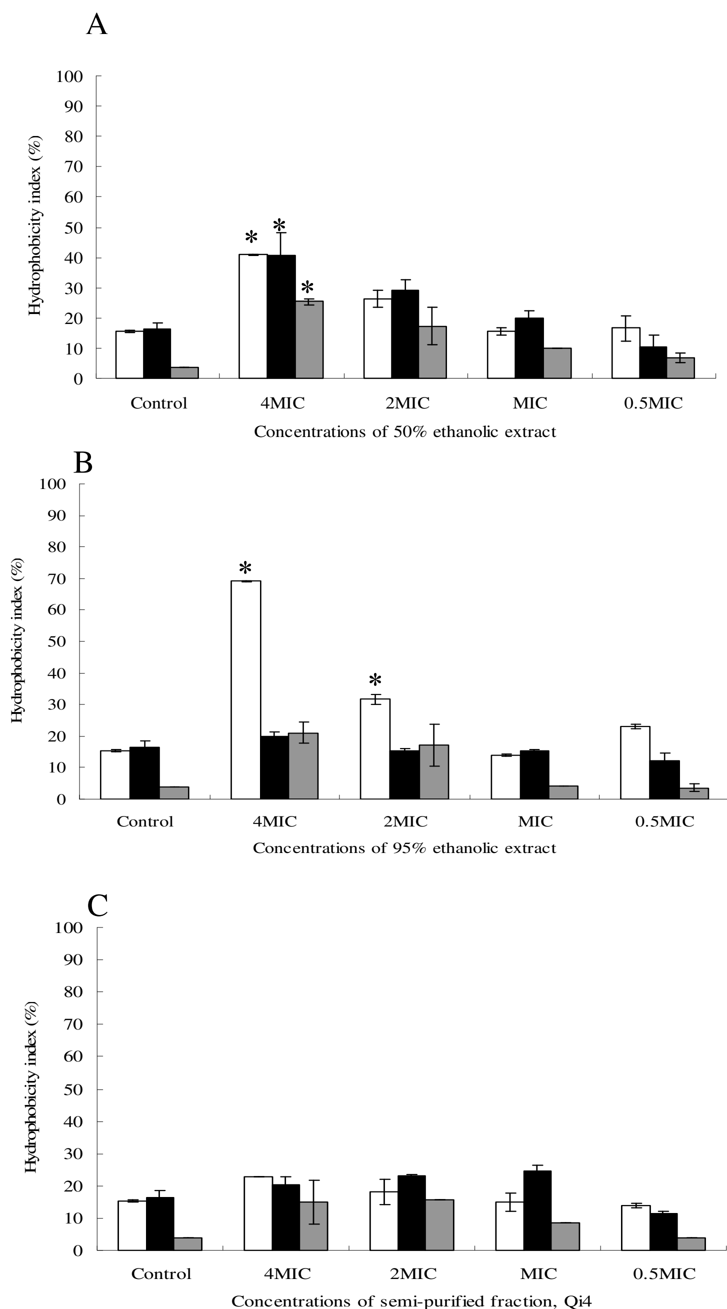


Figure 3.6 The mean hydrophobicity index at 18 h, as measured by the percentage of adhesion to toluene, of non-O157 treated with (A) 50% ethanolic extract, MIC = 1.56, 0.78, and 1.56 mg/ml, (B) 95% ethanolic extract, MIC = 0.78, 0.19, and 0.78 mg/ml, (C) semi-purified fraction, Qi 4, MIC = 250, 500, and 500 µg/ml. (□ = *Escherichia coli* O26: H11; ■ = *Escherichia coli* O111: NM; ▩ = *Escherichia coli* O22, and control = 1% DMSO). The *asterisks* indicate $P<0.05$ vs. control, by ANOVA and Fisher's least-significant differences (LSD) test.

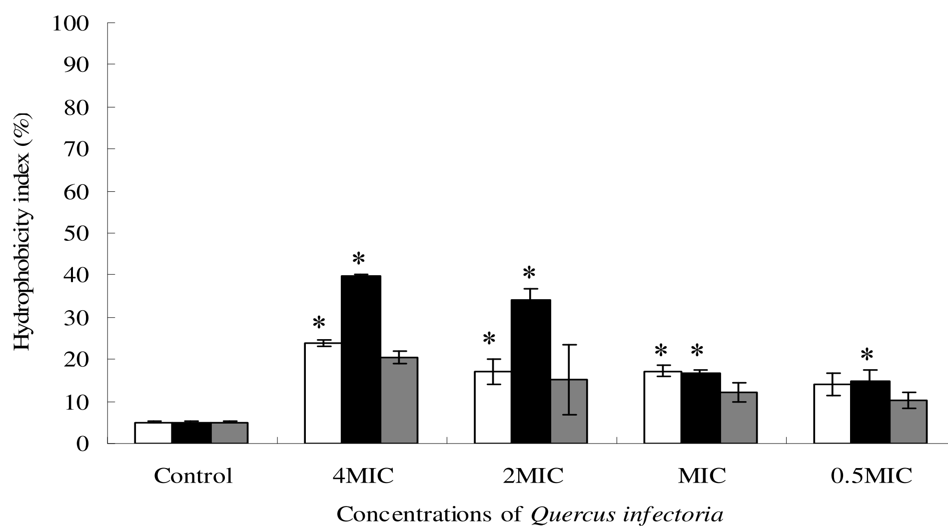


Figure 3.7 The mean hydrophobicity index at 18 h, as measured by the percentage of adhesion to toluene, of *Escherichia coli* ATCC 25922 treated with 50% ethanolic extract, MIC = 0.39 mg/ml (□), 95% ethanolic extract, MIC = 0.78 mg/ml (■), semi-purified fraction, Qi 4, MIC = 500 µg/ml (■), and control = 1% DMSO. The *asterisks* indicate $P < 0.05$ vs. control, by ANOVA and Fisher's least-significant differences (LSD) test.

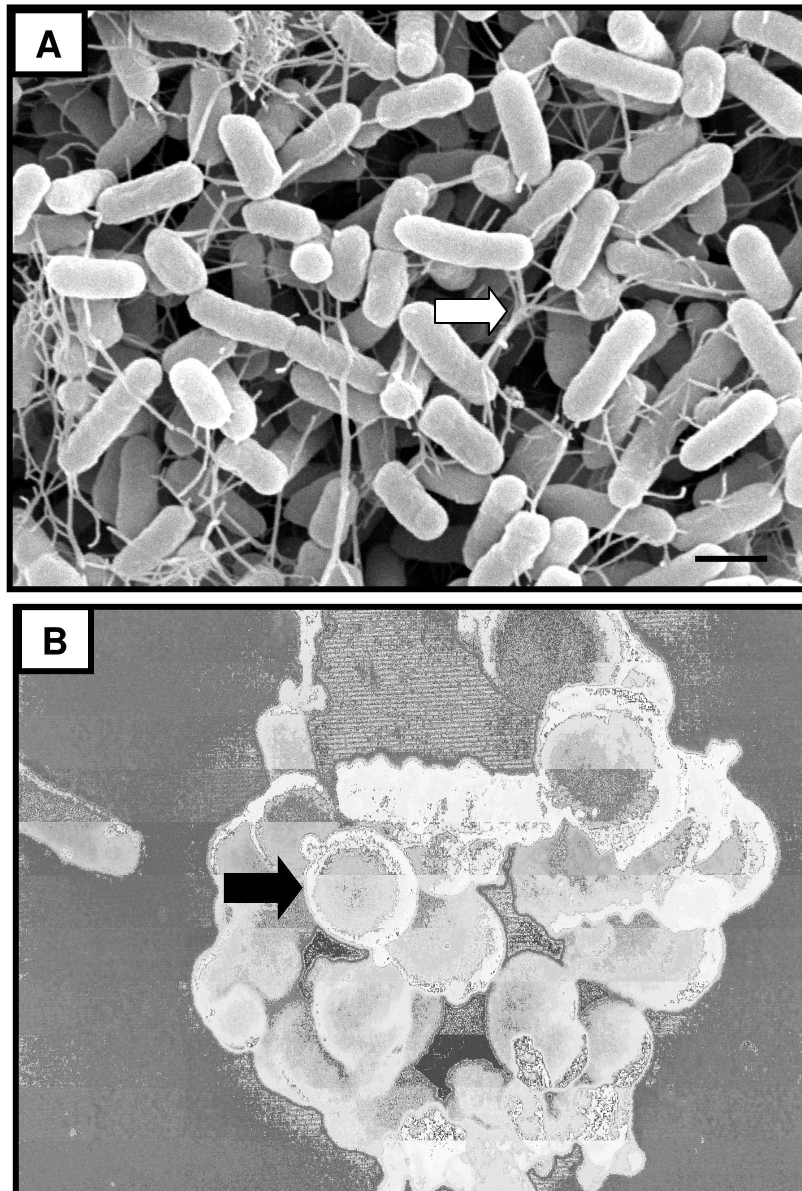


Figure 3.8 SEM micrographs of *Escherichia coli* O157: H7 at 4 h, Figure 3.8B, the bacterial cells treated with 50% ethanolic extract at 4MIC (3.12 mg/ml) showed coccoid form (arrow). Figure 3.8A, cells treated with 1% DMSO (control) showed rod-shapes form and peritrichous flagella (arrow). Scale bar 1 cm = 1 μm.

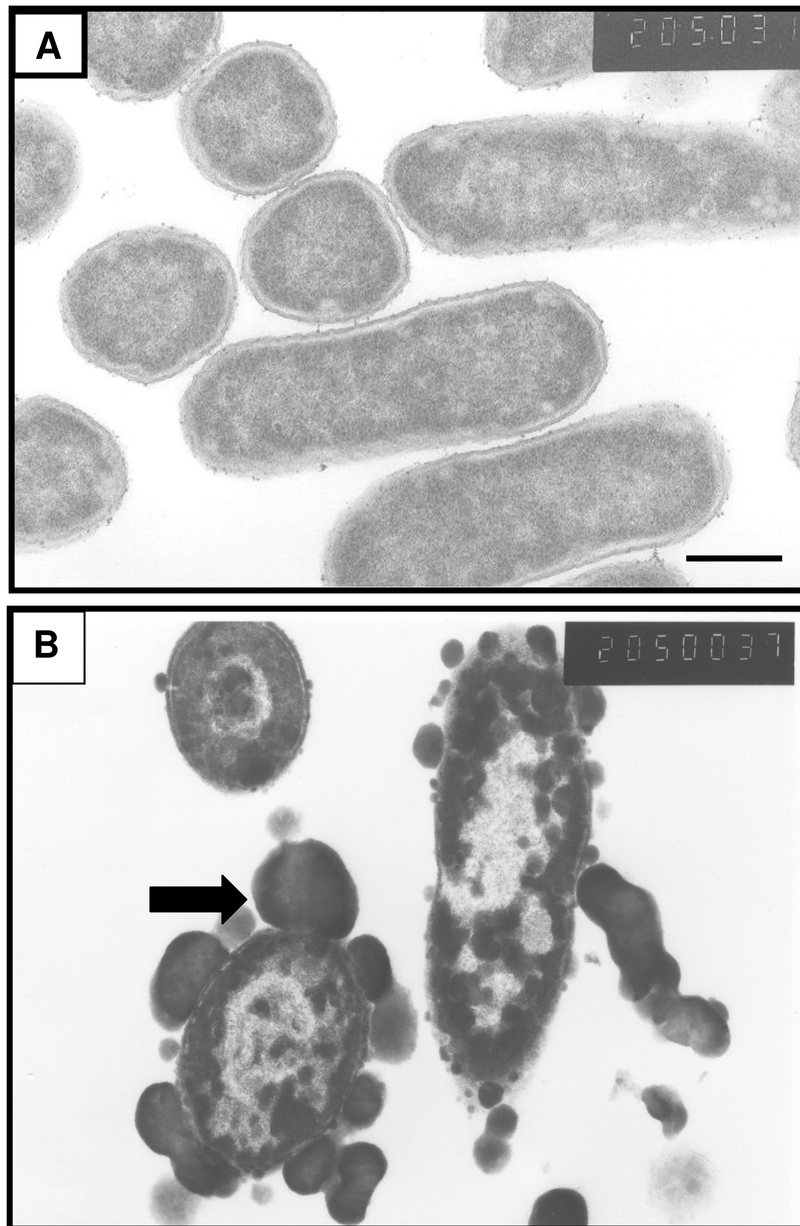


Figure 3.9 TEM micrographs of *Escherichia coli* O157: H7 at 4 h, Figure 3.9B, the bacterial cells treated with 50% ethanolic extract at 4MIC (3.12 mg/ml) revealed blebs on the cell wall surface (arrow). Figure 3.9A, cells treated with 1% DMSO (control) showed typical rod shapes, double layers of outer membrane closely apposed to the cytoplasmic membrane, and dispersed nuclear material. Scale bar 1.5 cm = 1 μm.

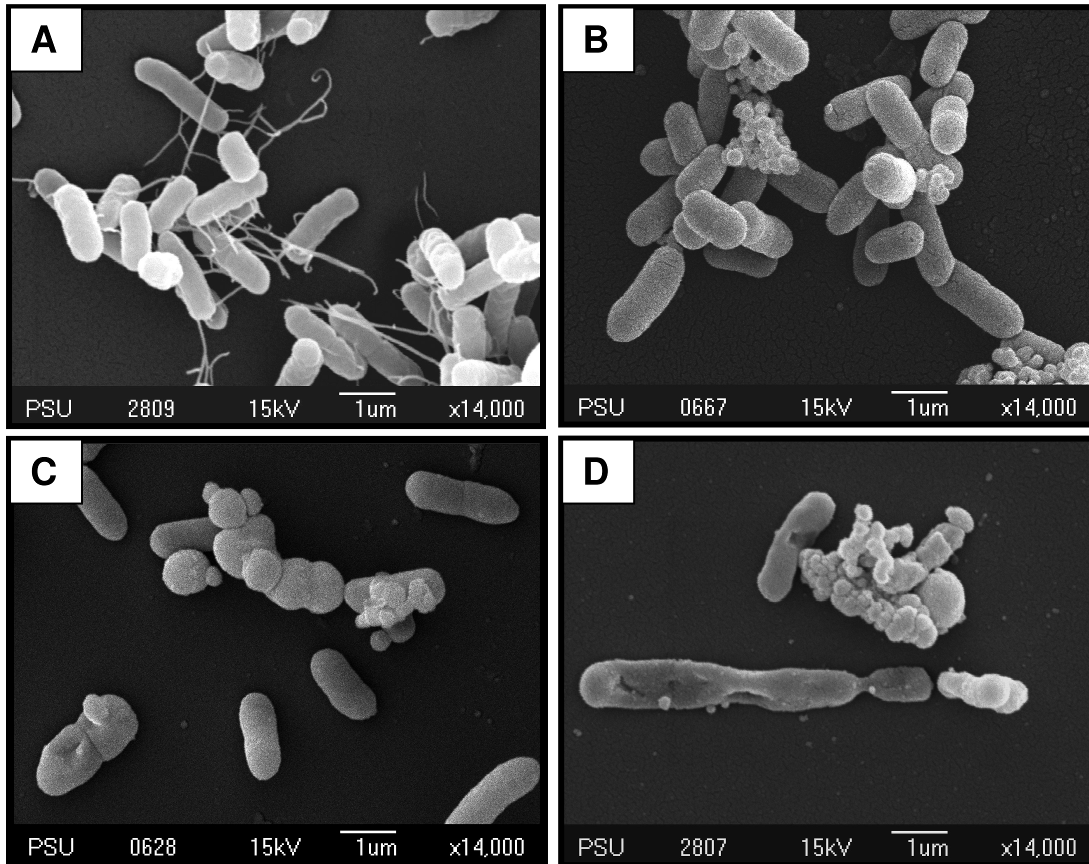


Figure 3.10 SEM micrographs of *Escherichia coli* O157: H7 treated with the 50% ethanolic extract of nut galls at 12 h, (Figure 3.10B to Figure 3.10C), the cells treated with the extract at MIC (0.78 mg/ml), 2MIC (1.56 mg/ml), showed abnormal cell division and loss of flagella. Figure 3.10D, the cells treated with the extract at 4MIC (3.12 mg/ml), demonstrated distortion and collapse. Figure 3.10A, the cells treated with 1% DMSO (control) showed typical rod shape, smooth cell surfaces and peritrichous flagella, Scale bar 1 cm = 1 μm.

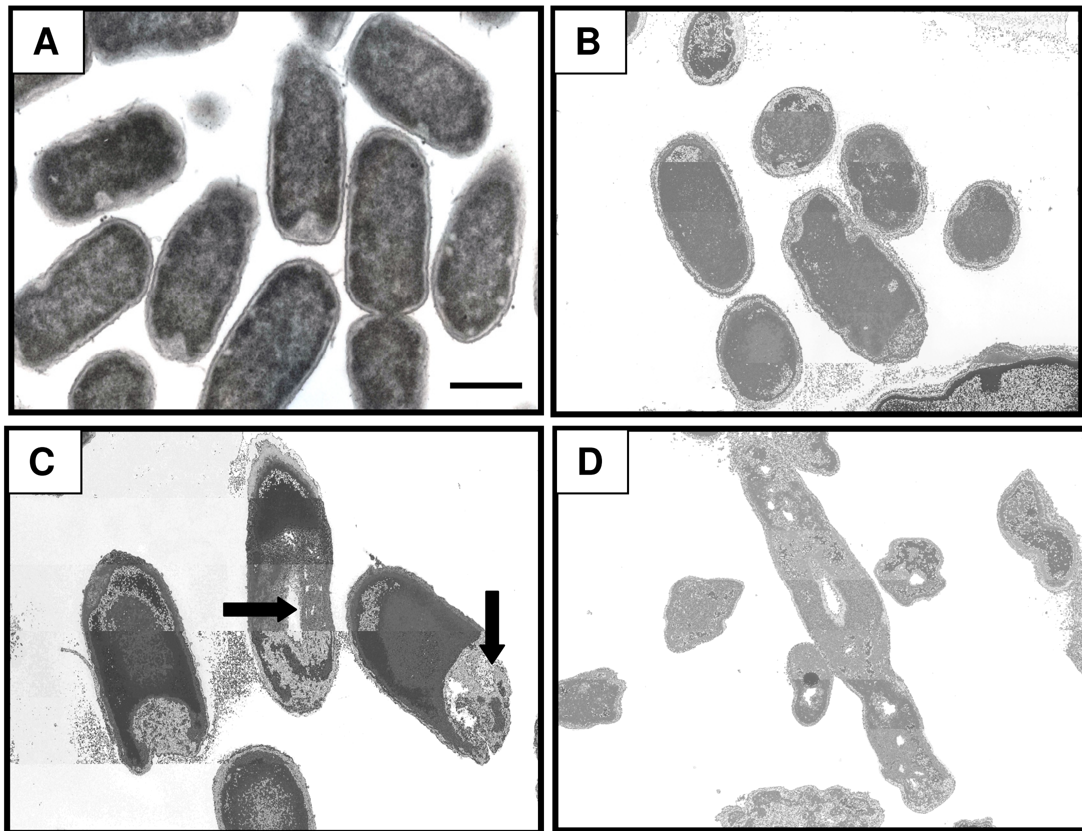


Figure 3.11 TEM micrographs of *Escherichia coli* O157: H7 treated with the 50% ethanolic extract of nut galls at 12 h. Figure 3.11B, at MIC (0.78 mg/ml), there was no evidence of membrane damage or release of intracellular contents. Figure 3.11C, at 2MIC (1.56 mg/ml), shows disruption in the outer and cytoplasmic membranes at the polar regions of the cells, loss of the double membranes structure, and some vacuolizations were seen (arrows). Figure 3.11D, at 4MIC (3.12 mg/ml), cells also demonstrated distortions and collapse of the cell membranes, and fragmentation of the bacteria. Figure 3.11A, cells treated with 1% DMSO (control) showed typical rod shape, double layers of outer membrane closely apposed to the cytoplasmic membrane, and dispersed nuclear material. Scale bar 1 cm = 0.7 μm.

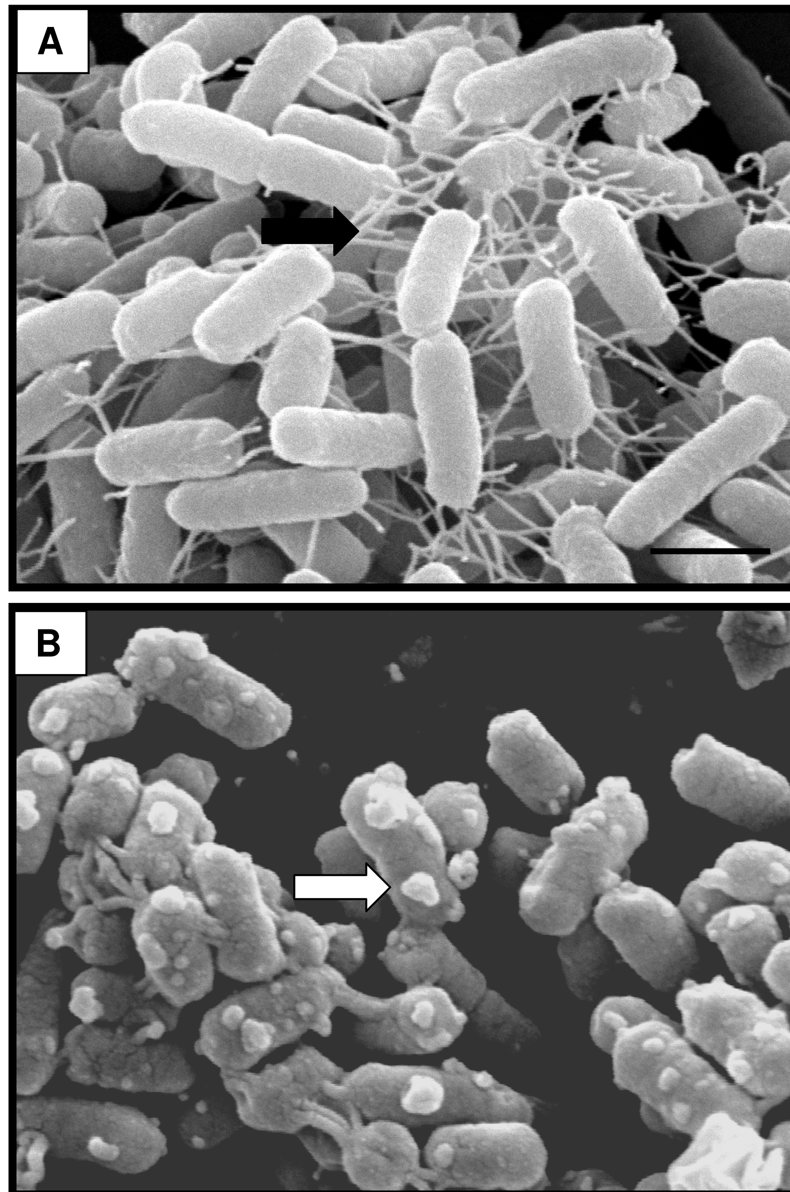


Figure 3.12 SEM micrographs of *Escherichia coli* O157: H7 at 12 h, Figure 3.12B, the bacterial cells treated with semi-purified Qi 4 fraction at MIC (250 μg/ml) demonstrating blebs on cell wall surface (arrow). Figure 3.12A, cells treated with 1% DMSO (control) showing typical rod shapes and smooth cell surfaces with peritrichous flagella (arrow). Scale bar 1.5 cm = 1 μm.

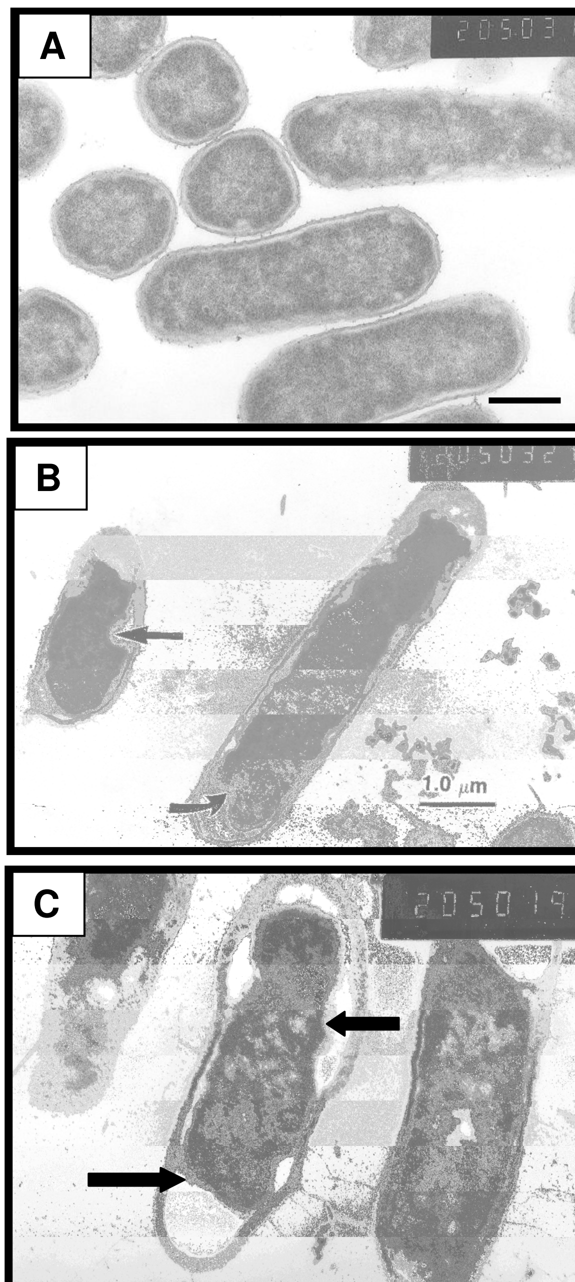


Figure 3.13 TEM micrographs of *Escherichia coli* O157: H7 and *Escherichia coli* ATCC 25922 treated with the semi-purified Qi 4 fraction after 12 h. Figure 3.13B, *Escherichia coli* O157: H7 and Figure 3.13C, *Escherichia coli* ATCC 25922 treated with the semi-purified Qi 4 fraction at MIC (250 μg/ml) showing detachment of the plasma membrane from the outer membrane (arrows). Figure 3.13A, cells treated with 1% DMSO (control) showed typical rod shapes, double layers of outer membrane closely apposed to the cytoplasmic membrane, and dispersed nuclear material. Scale bar 1 cm = 0.5 μm.

9. *In vivo* study on the effect of semi-purified Qi 4 fraction on infected mice

9.1 Determination of *E. coli* O157: H7 in faeces

IRC mice were inoculated intragastrically with $2x10^9$ cfu of *E. coli* O157: H7 RIMD 05091078, *E. coli* O157: H7 EDL 933 and treated with semi-purified Qi 4 fraction. The numbers of *E. coli* O157: H7 in faeces were examined for ten days (**Figure 3.14** and **Figure 3.15**). In control group without semi-purified Qi 4 fraction, the level of *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933 colonization in the faeces increased from day one to day ten. After 24 h, mice were administered with semi-purified Qi 4 fraction at a dose of MIC (0.25 mg/ml) and 2MIC (0.5 mg/ml), the pattern of colonization in the the faeces was similar to the control group. At a dose of 4MIC (1 mg/ml), viable bacteria were not detected in the faeces of the treated group after day five (**Figure 3.16**).

9.2 Determination of the number of *E. coli* O157: H7 in the gastrointestinal tract

The numbers of *E. coli* O157: H7 in small intestine, caecum, and colon of mice were examined at day ten following the inoculation. Both *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933 were detected in the caecum and colon of infected mice (**Table 3.8**). However, the organisms were not detected in the small intestine. When mice were treated with the semi-purified Qi 4 fraction 24 h post inoculation with *E. coli* O157: H7, the numbers of the bacteria in the caecum as well as the colon decreased to less than 100 cfu/g tissues at day ten.

9.3 Estimation of bacterial colonization in the gastrointestinal tract

Numbers of bacteria colonization in the small intestine, caecum, and colon are shown in **Table 3.9**. In some infected mice without semi-purified Qi 4 fraction treatment, the large amount of bacteria were seen on the caecum and colon. Minimal or moderate amount of colonized bacteria were presented in caecum and colon at MIC (0.25 mg/ml) and 2MIC (0.5 mg/ml). There was very few bacterial colonization observed in the caecum and colon at 4 MIC (1 mg/ml).

9.4 Histopathological examination

To determine histopathological changes following treatment with semi-purified Qi 4 fraction, all mice were sacrificed on day ten. Tissues from their caecum **(Figure 3.17** and **Figure 3.18)**, colon **(Figure 3.19** and **Figure 3.20)**, and kidneys **(Figure 3.21, Figure 3.22,** and **Figure 3.23)** were examined histopathologically.

Microscopic examination of the caecum and colons removed from infected mice revealed colonization at caecal lumen (Figure 3.18A), and the luminal area of colon (Figure 3.20A). The caecal and colon mucosa of a mouse infected with EHEC and treated with semi-purified Qi 4 fraction at MIC (0.25 mg/ml) (Figure 3.18B), (Figure 3.20B), 2MIC (0.5 mg/ml) (Figure 3.18C), (Figure 3.20C) had numbers of colonized bacterial cells that were similar to those in Figure 3.18A and Figure 3.20A. Of interest was the finding that the caecal (Figure 3.18D) and colon (Figure 3.20D) mucosa of a mouse infected with EHEC and treated with semi-purified Qi 4 fraction at 1 mg/ml daily for ten days had lower numbers of colonizied bacteria, compared to the control mice (Figure 3.17 and Figure 3.19). Kidney from control mice showed glomerulus with normal histology (Figure 3.21). A enlarged glomerulus and a marked increase in mesangial cells and the mesangial matrix were observed in sections of kidneys removed from infected mice (Figure 3.22A). The glomerulus from the mice treated with the semi-purified Qi 4 fraction-treated mice showed markedly less mesangial cells than were present in the glomerulus of the infected group (Figure 3.22C). Treatment of the mice with semi-purified Qi 4 fraction (1 mg/ml) revealed decreased in mesangial cells and normal glomerulus (Figure 3.22D). Ultrastructural examination of kidney from mice infected with *E. coli* O157: H7 demonstrated a marked increase in mesangial cells and the mesangial matrix (Figure 3.23B), with the endothelial cells showing irregular borders with cytoplasmic bleb formation (Figure 3.23C). In contrast, in the kidney of mice infected with *E. coli* O157: H7 and treated with semi-purified Qi 4 fraction at 1 mg/ml (Figure 3.23D), the endothelial cells that line the capillary lumen had a normal morphology. Control of glomerulus showed normal mesangial cells, mesangial matrix, and capillary lumen (Figure 3.23A).

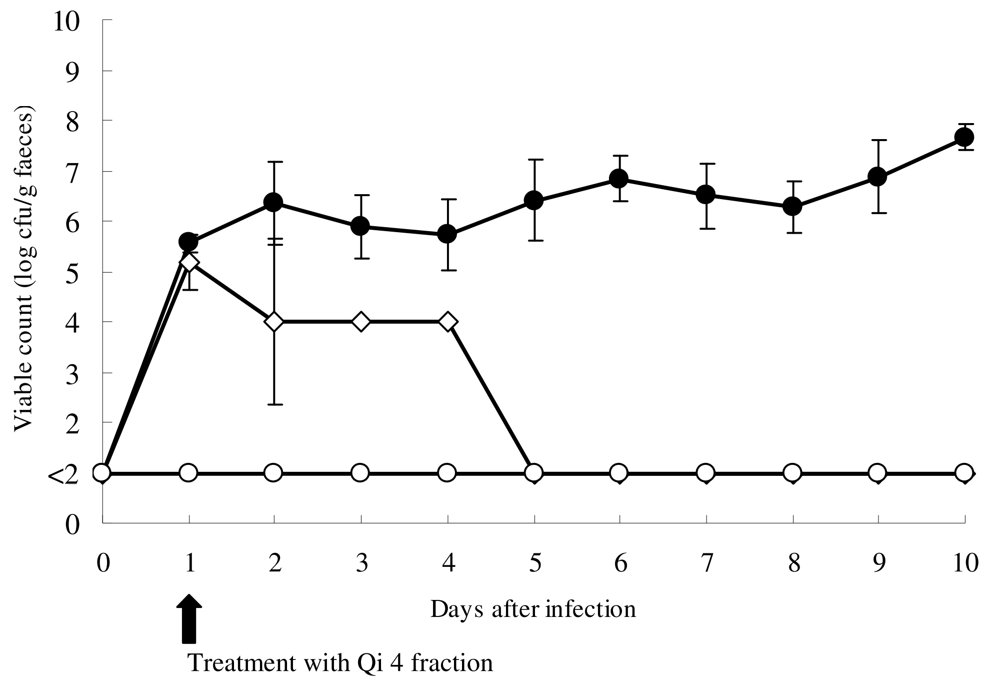


Figure 3.14 Numbers of *Escherichia coli* O157: H7 RIMD 05091078 in faeces. Mice pretreated with streptomycin, followed by intragastrically inoculation of 2x10^9 cfu (●). Mice treated with semi-purified Qi 4 fraction at a dose of 1 mg/ml (◇), control PBS (○). Results are expressed as means ± the standard deviations for groups of five animals. Limit of detection was100 cfu/g faeces.

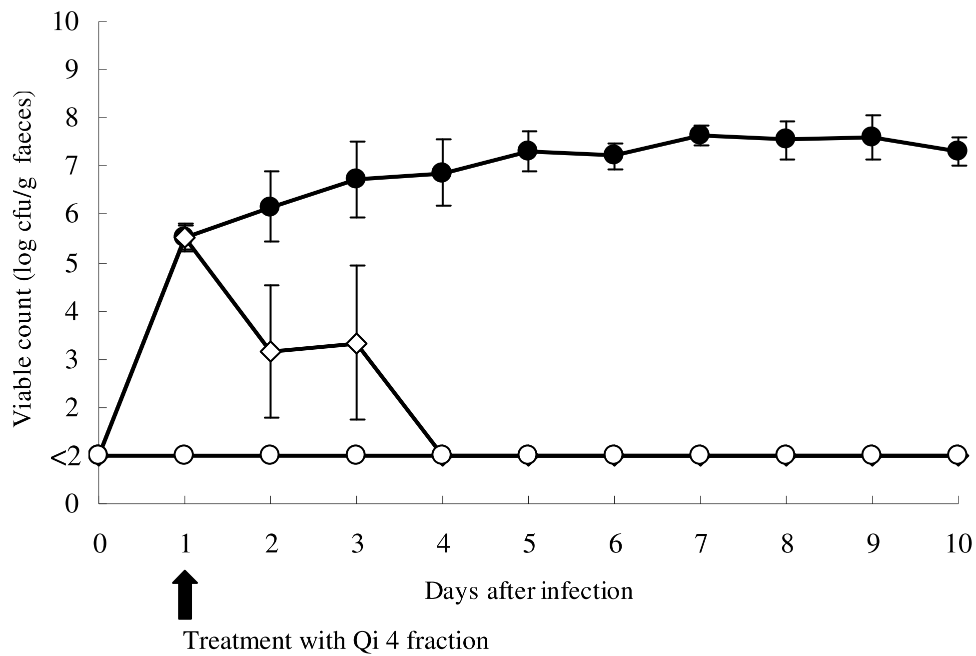
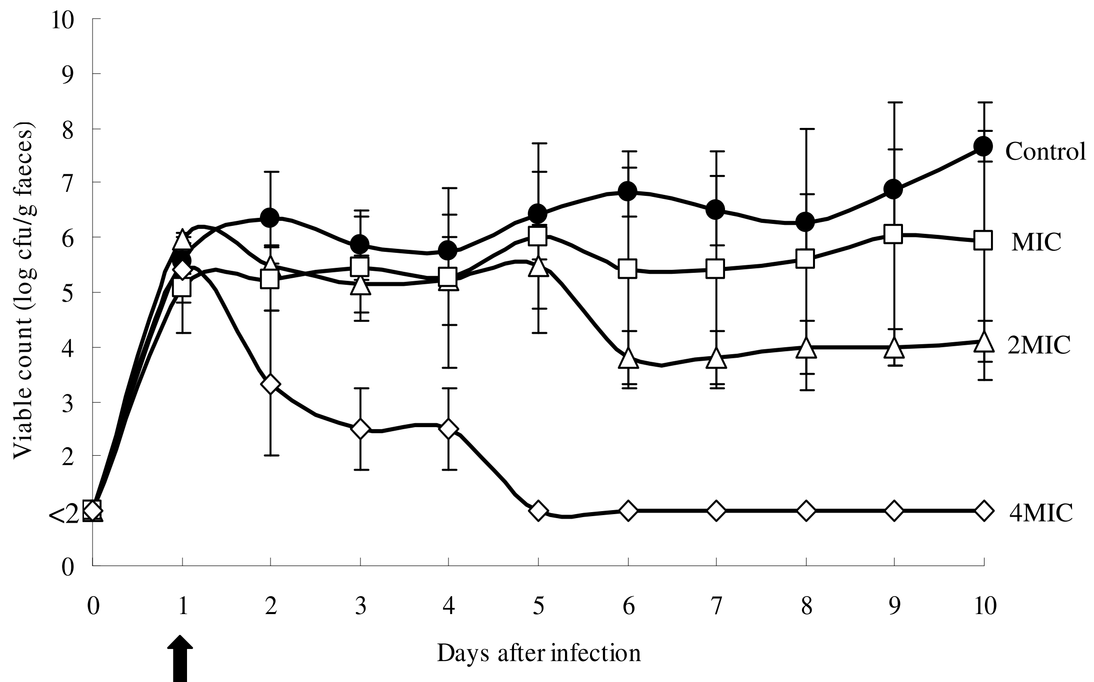


Figure 3.15 Numbers of *Escherichia coli* O157: H7 EDL 933 in faeces. Mice pretreated with streptomycin, followed by intragastrically inoculation of $2x10^9$ cfu (●). Mice treated with semi-purified Qi 4 fraction at a dose of 1 mg/ml (◇), control PBS (○). Results are expressed as means ± the standard deviations for groups of five animals. Limit of detection was 100 cfu/g faeces.



Treatment with Qi 4 fraction

Figure 3.16 Dose-dependent efficacy of semi-purified Qi 4 fraction to reduce bacterial numbers when administered 24 h after mice were intragastrically infected with 2×10^9 cfu of *Escherichia coli* O157: H7 RIMD 05091078, □ = MIC (0.25 mg/ml), △ = 2MIC (0.5 mg/ml), ◇ = 4MIC (1 mg/ml), ● = (Control). Results are expressed as means ± the standard deviations for group of five animals. Limit of detection was 100 cfu/g faeces

Table 3.8 Viable bacterial counts of *Escherichia coli* O157: H7 in the gastrointestinal tract of enterohaemorrhagic *Escherichia coli* infected mice (n = 5).

E. coli Strains	Inoculum size	Treatment	Viable count (log cfu/g tissue) at day 10		
			small intestine	caecum	colon
E. coli O157: H7 (RIMD 05091078)	2 x10^9	-	<2	7.2±2.0	8.7±1.0
E . coli O157: H7 (EDL 933)	2 x10^9	-	<2	8.7±1.2	9.5±1.2
E . coli O157: H7 (RIMD 05091078)	2 x10^9	Qi 4 (4 MIC)	<2	<2	<2
E. coli O157: H7 (EDL 933)	2 x10^9	Qi 4 (4 MIC)	<2	<2	<2
Control	-	PBS	<2	<2	<2

Data are represented by $\log_{10}$ cfu/g tissue and the means ± standard error.

Limit of detection was 100 cfu/g tissue.

Table 3.9 Histological examination of numbers colonized bacteria in the gastrointestinal tract of mice (n = 5) at day ten after inoculation with *Escherichia coli* O157: H7 RIMD 05091078 with and without treated with semi-purified Qi 4 fraction.

Tissue	Control	MIC	2MIC	4MIC
Small intestine	0.3	0.3	0.2	0.10
Caecum	3.0	2.8	2.0	0
Colon	3.0	1.75	1.2	0.80

0 = none, 1 = low, 2 = moderate, 3 = high.

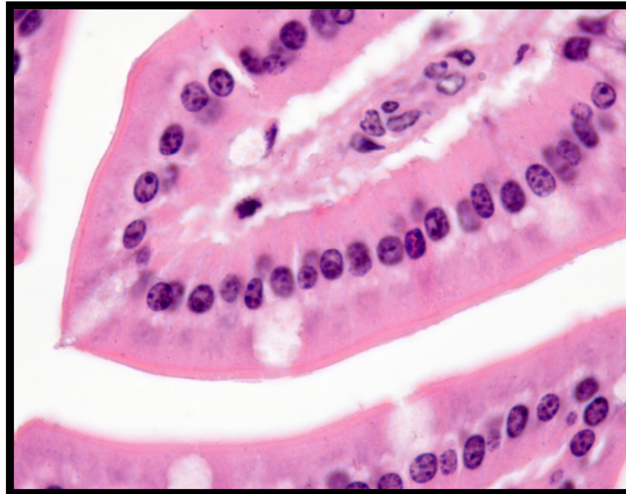


Figure 3.17 Light microscopy of the caecal mucosa of mice at day ten. Control mice had a normal mucosal epithelium without bacterial cells. Original magnification, X1000.

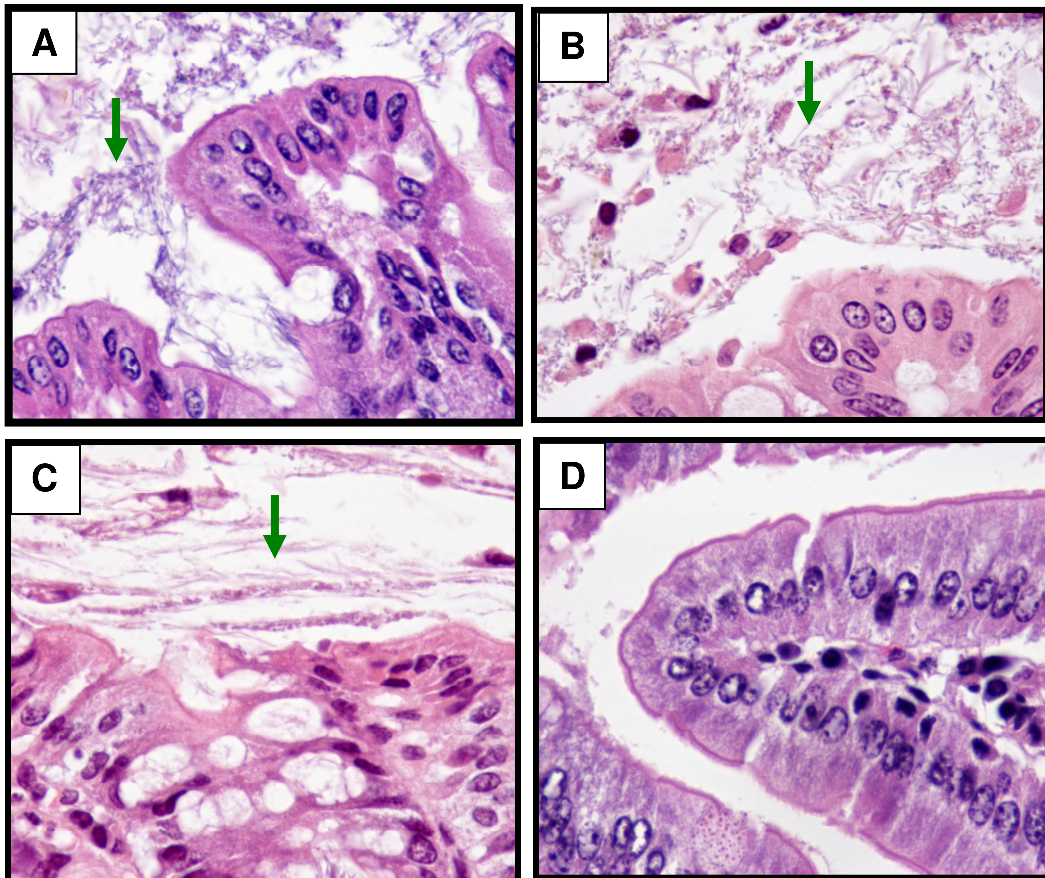


Figure 3.18 Light microscopy of the caecal mucosa of mice at day ten. The caecal mucosa of a mouse infected intragastrically with *Escherichia coli* O157: H7 showed colonization of bacterial cells in the luminal (arrow) (Figure 3.18A). The caecal mucosa of a mouse infected with EHEC and treated with semi-purified Qi 4 fraction at MIC(0.25 mg/ml) (Figure 3.18B), 2MIC (0.5 mg/ml) (Figure 3.18C) daily for ten days had numbers of colonized bacterial cells (arrow) that were similar to those in Figure 3.18A. When the mice received semi-purified Qi 4 fraction at 4MIC (1 mg/ml) (Figure 3.18D), numbers of colonized bacterial cells decreased, compared to the control level. Original magnification, X1000.

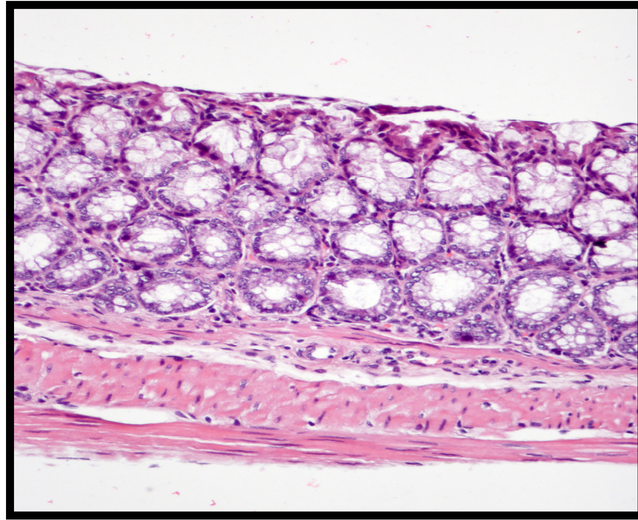


Figure 3.19 Light microscopy of the colon mucosa of mice at day ten. A control mouse showed normal mucosal epithelium without bacteria cells. Original magnification, X200.

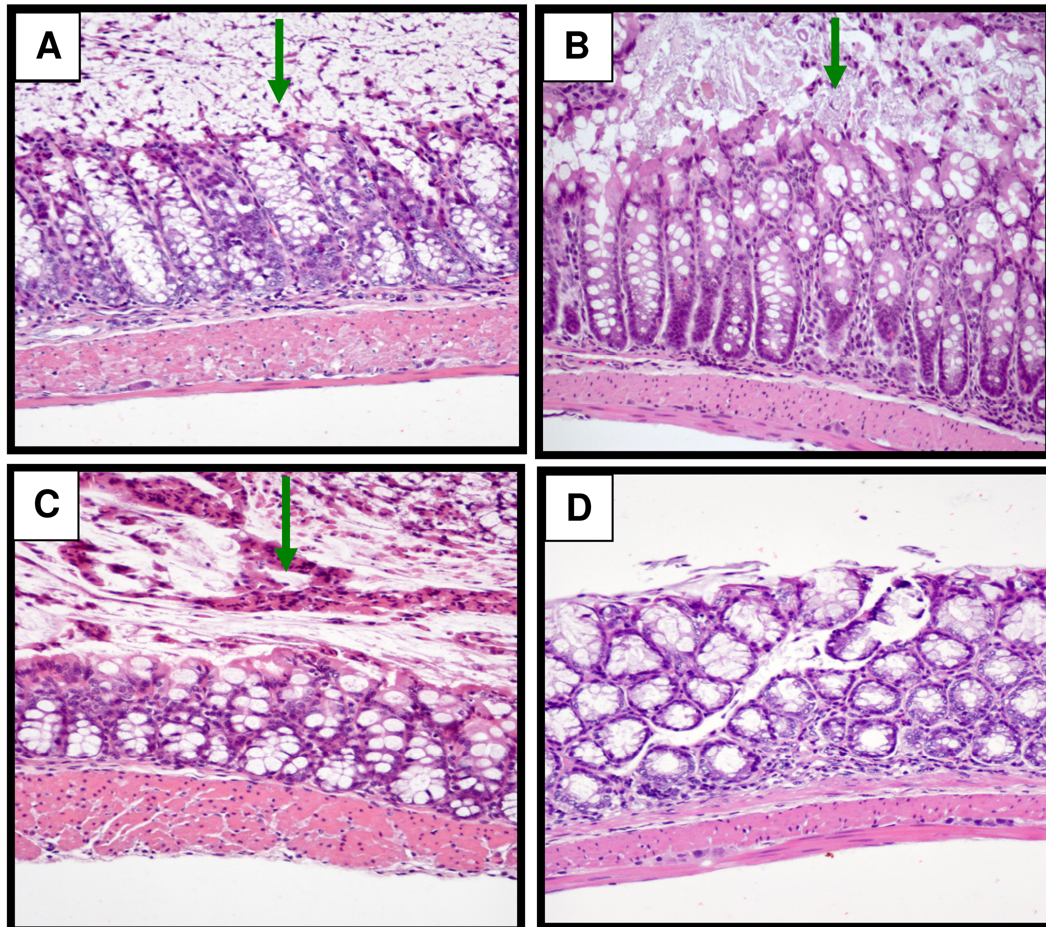


Figure 3.20 Light microscopy of the colon mucosa of mice at day ten. The colon mucosa of a mouse infected intragastrically with *Escherichia coli* O157: H7 showed colonization of bacterial cells in the luminal area (arrow) (Figure 3.20A). The colon mucosa of a mouse infected with EHEC and treated with semi-purified Qi 4 fraction at MIC (0.25 mg/ml) (Figure 3.20B), 2MIC (0.5 mg/ml) (Figure 3.20C) daily for ten days had numbers of colonized bacterial cells (arrow) that were similar to those in Figure 3.20A. When the mice received semi-purified Qi 4 fraction at 4MIC (1 mg/ml) (Figure 3.20D), with the numbers of colonized bacterial cells being decreased compared to the control level. Original magnification, X200.

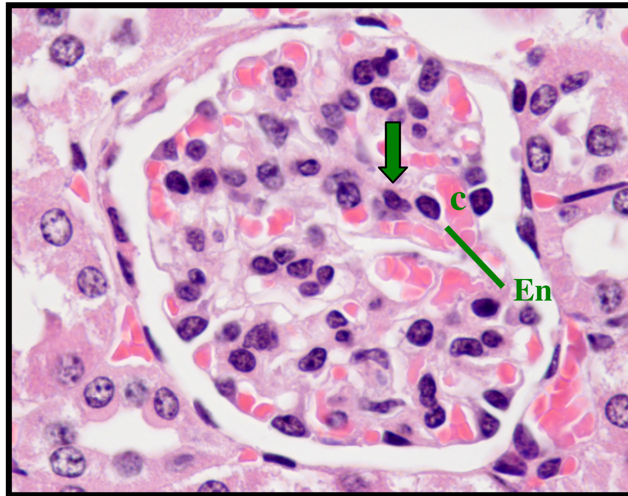


Figure 3.21 Light microscopy of the glomerulus of mice at day ten. Control mice revealed normal mesangial cells (arrow), (C = capillary lumen, En = endothelial cell). Original magnification, X1000.

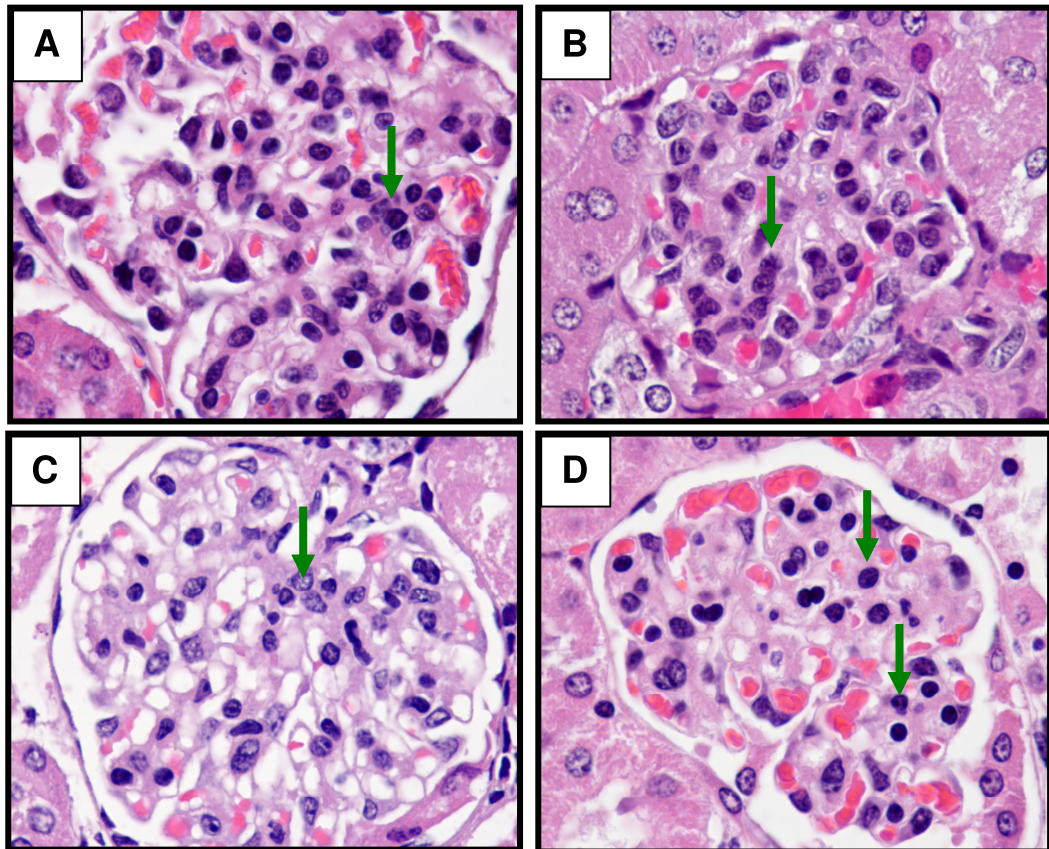


Figure 3.22 Light microscopy of the glomerulus of mice at day ten. The enlarged glomerulus of mice intragastrically infected with *Escherichia coli* O157: H7 demonstrated a marked increase in mesangial cells and mesangial matrix (arrows) (Figure 3.22A). A glomerulus of mice infected with EHEC and treated with semi-purified Qi 4 fraction at MIC (0.25 mg/ml) (Figure 3.22B), 2MIC (0.5 mg/ml) (Figure 3.22C) showed a marked increase in mesangial cells and mesangial matrix (arrow). A glomerulus of mice infected with EHEC and treated with semi-purified Qi 4 fraction at 4MIC (1 mg/ml) (Figure 3.22D) demonstrated the numbers of mesangial cells decreased (arrows) and normal glomerulus. Original magnification, X1000.

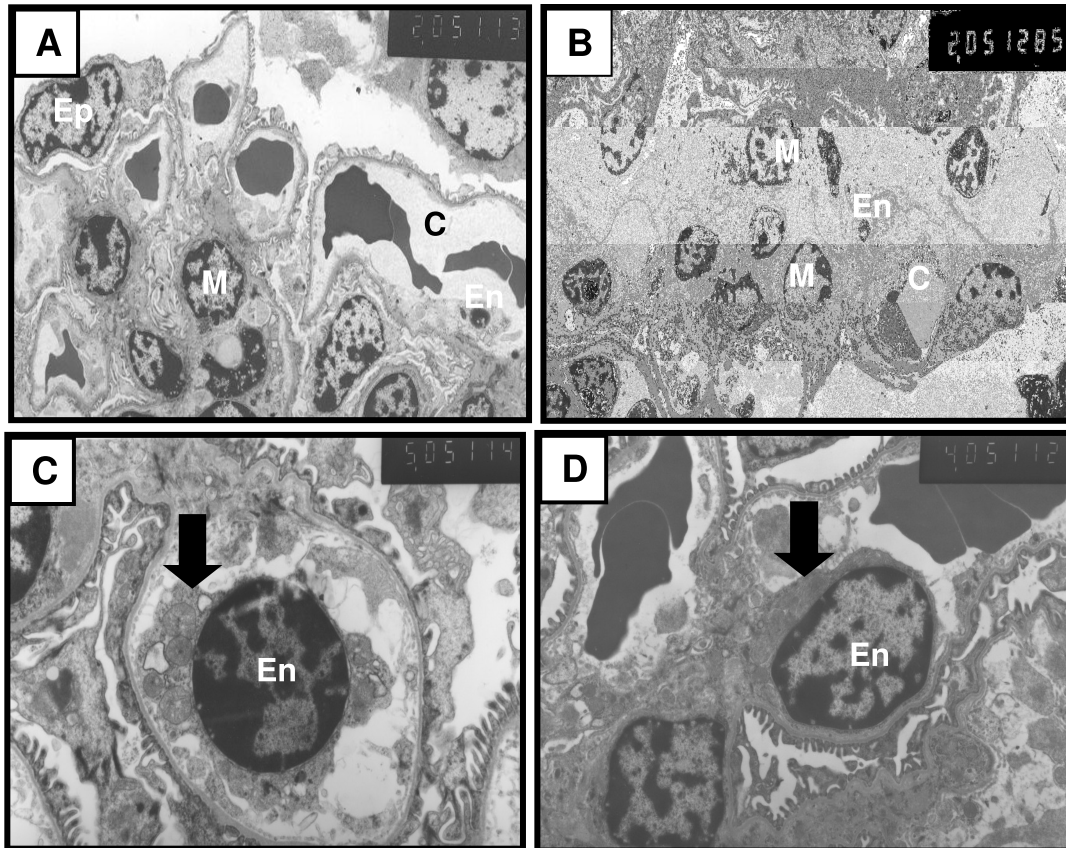


Figure 3.23 TEM micrographs of the glomerulus of mice at day ten. Mice intragastrically infected with *Escherichia coli* O157: H7 demonstrated a marked increase in mesangial cells and mesangial matrix (Figure 3.23B), X2000, and the endothelial cells showed irregular borders with cytoplasmic bleb formation, (arrow) (Figure 3.23C), X5000. Mice infected with *Escherichia coli* O157: H7 and treated with semi-purified Qi 4 fraction at 4MIC (1 mg/ml) (Figure 3.23D), X5000, revealed endothelial cells lining the capillary lumen with normal morphology (arrow). Control mice revealed normal mesangial cells (M), (C = capillary lumen, Ep = epithelial cell, En = endothelial cell) (Figure 3.23A), X 2000.

CHAPTER 4

DISCUSSION

1. Antibacterial activities of semi-purified Qi 4 fraction of nut galls against enterohaemorrhagic *E. coli* O157: H7

Many medicinal plants produce antibacterial compounds that act against Gram-positive bacterial while very few of these compounds are active against Gram-negative bacteria. It is therefore of much interest that we have isolated a compound from nut galls that acts on EHEC isolates. It has been shown previously that polyphenols obtained from medicinal plants can inhibit bacteria that cause food-borne diseases. Green tea, one plant commonly used in medicine contains mostly condensed tannins such as epigallocatechin (EGC) and epigallocatechin-*3*-*O*-gallate (EGCg) (Taguri *et al*., 2006). However, little is known about the role of the hydrolyzable group. An ethanolic extract of nut galls has been reported to contain mainly hydrolyzable tannins of the gallic acid type (Pithayanukul *et al*., 2005). The reaction of both hydrolyzable and condensed tannins with proteins depends on the spatial configuration of the molecules and availability of the reactive phenolic groups. There are several hypotheses on the antibacterial activity of polyphenols. For example, Ikigai *et al*., (1993) demonstrated that bactericidal catechins primarily act on and damage bacterial cell membranes. Arakawa *et al*., (2004) suggested that oxidative polyphenols generate hydrogen peroxide, which may mediate antibacterial activity.

In addition to the antibacterial activity of the semi-purified Qi 4 fraction described in this study, its inhibitory effect on VT1 and VT2 production by the *E. coli* O157: H7 RIMD 05091078 at 20 h was clearly demonstrated. This is of interest as it is well documented that VT can cause vascular endothelial damage in HC and HUS patients (Richardson *et al*., 1988). The Verocytotoxicity assay further confirmed that fraction Qi 4 primarily inhibited the release of VT, resulting in significantly higher cell recovery than that of control ($P < 0.05$). In contrast, the administration of certain antimicrobial agents for the treatment of EHEC infection has

been previously reported to result in the increased level of VT production (Yoh and Honda, 1997; Yoh *et al*., 1999). Verocytotoxins are holotoxins composed of a single enzymatic A subunit of approximately 32 kDa in association with a pantamer of receptor-binding B subunit of 7.7 kDa (Iijima and Honda, 1997). The expression of the A-and B-subunit genes differently regulated the production of Verocytotoxin which is negatively regulated at the transcriptional level by an iron-sulfur protein corepressor complex (O'Brien *et al*., 1992). Plant extracts might act directly or indirectly, interfere with the transcription and/or translational steps, and reduce the production of the toxins (Sakagami *et al*., 2001). The biological activities of VT1 and VT2 have been reported to be different. It has been proved that VT1 and VT2 are apparently different in both their secretion systems and their gene regulation systems (O'Brien and Holmes, 1987). VT2 was 1000-fold more active on human renal endothelial cells than VT1 (Louise and Obrig, 1995), and VT2 also had a 50% lethal dose that was 400 times lower than that of VT1 when injected intravenously or intraperitoneally into mice (Tesh *et al*., 1993). Many other factors such as growth conditions including composition of medium, addition of antibiotic presence, and aeration can affect the production level of the toxins or release of the toxins outside the cells (Ito *et al*., 1997).

2. Morphological and ultrastructural changes in the cell structure of enterohaemorrhagic *E. coli* O157: H7 following treatment with nut galls

These observation indicate that treatment with an ethanolic extract of nut galls might have interfered with the bacterial cell division, leading to cell deformation the typical short rods to long rod shapes. These finding are consistent with the results of a previous study which examined epigallocatechin gallate (EGCG) on food-borne pathogens (Si *et al*., 2006). From the SEM observations it appears that, after loss of contents, *E. coli* O157: H7 cells treated with 4MIC of extract collapsed, which enable them to pass more easily through the pores of the membrane. This is in accordance to result of (Lambert *et al*., 2001), who observed *Origanum vulgare* disrupted bacterial cell membranes permeability. From the TEM micrographs such as elongation of cells, vacuoles in cytoplasm, and loss of cell integrity due to 2MIC and

4MIC exposure were also observed. Vaara, (1992) explained that the outer membrane of Gram-negative bacteria is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its lipopolysaccharides (LPS) covered surface. The observed effects of these ethanolic extract are believed to be caused by disruption membrane structure. Recent work, on the antimicrobial activity of beery compounds against *Vibrio parahaemolyticus* and *E. coli* O157: H7 was correlated with highest ellagic acid concentration and antioxidant activity (Puupponen-Pimia *et al*., 2005). The two suggest mechanisms of action were disruption of the plasma membrane due to localized hyper-acidification, and disruption of the membrane-transport and electron transport. We would like to suggest that the mechanisms of action of our ethanolic extract against *E. coli* O157: H7 are weakening of the membrane by the highest ellagic acid concentration and the antioxidant activity present in the nut galls extract, a view supported by our preliminary experiments. However this requires further confirmation. Transmission electron microscopy reveals that some of the dead cells still appear to have an intact cell walls structure. This information suggests that autolysis is a secondary event that occurs after ethanolic extract had induced cell death.

3. Modifications of cell surface hydrophobicity of Shiga toxigenic *E. coli* by nut galls extract

Many workers have studied cell surface hydrophobicity of *E. coli* with a limited number of strains, usually targeted the serotype O157 (Hassan and Frank, 2004; Ryu *et al*., 2004). Although the serotype O157: H7 is the most predominant isolate from significant outbreaks, other serotypes including O111 and O26 have also been involved (OzFoodNet *et al*., 2004). In the present study, we evaluated the *in vitro* modifications of cell surface hydrophobicity by nut galls extracts on a variety of STEC isolates. MIC values for different STEC strains in the presence of the extracts at different concentrations of solvent have been presented in Table **3.4** and Table **3.5.** The semi-purified Qi 4 fraction had the biggest yield and had the best MIC & MBC, therefore this fraction was used for further studies in the MATH experiment. Diseases of the digestive tract caused by bacterial infection are usually the result of the adherence of the bacteria to host cells and subsequently their proliferation leading

to colonization. The initial adhesion of bacteria to some surfaces may rely on hydrophobic interactions (Briandet *et al*., 1999). High cell surface hydrophobicity appears to be important in the pathogenesis of several microorganisms associated with gastrointestinal infections (Lachica, 1990). Adhesive strains often possess high cell surface hydrophobicity as determined by the salt aggregation test (SAT) and other methods (Busscher and Weerkamp, 1987). *Escherichia coli* has a net negative surface charge and a low cell surface hydrophobicity as compared with many other bacteria (Rivas *et al*., 2006a). From **Figure 3.5** and **Figure 3.6**, the HPBI of STEC strains were found to be by far less than 70%, which indicated the hydrophilic nature of the strains. We suggest that the low cell surface hydrophobicity of these strains may allow the pathogens to attach and penetrate the gastric mucous layer. The values of HPBI of EHEC strains as well as *E. coli* ATCC 25922 (**Figure 3.7**) at sub-MICs of the extract did not show definite patterns. In contrast the growth of EHEC cells in the presence of 4MIC levels of nut galls extracts resulted in moderate surface hydrophobicity (but the HPBI was still lower than 70%). This may facilitate phagocytosis of the host. Other workers have reported the loss of capsular (K) and lipopolysaccharide (O) antigens resulted in increased surface hydrophobicity and subsequently susceptibility to phagocytosis (Williams *et al*., 1988).

Some studies have found a correlation between the surface hydrophobicity of *E. coli* and attachment to surface (Benito *et al*., 1997). In contrast, other studies have reported no correlation between EHEC hydrophobicity or cell surface charge and attachment to surface (Hassan and Frank, 2004; Rivas *et al*., 2005; Rivas *et al*., 2006a). A limited study has reported no modification on cell surface hydrophobicity from nut galls extract using the SAT test. The contradictory nature of the findings may be due to the influence of many different substrates and methods used to quantify attachment. In complex issues such as pathogenicity, bacterial hydrophobicity is likely to be one of many parameters which determine the ability of cells to adhere, invade, and cause damage. We propose that nut galls extract modulates the first mucosal surface contact phase of infection by enhancing cell surface hydrophobicity. Further studies are required to evaluate the biological effects of nut galls extract in conditions similar to those found *in vivo*. The information may contribute to the understanding of EHEC attachment to host intestinal mucosal

surface and assist in the implementation of alternative treatment and prophylaxis control of these pathogens in modern medicine.

4. Colonization and pathological changes due to enterohaemorrhagic *E. coli* O157: H7 in mice

Enterohaemorrhagic *E. coli* O157: H7 has not been found to be invasive (Mc Kee *et al*., 1995), and it has therefore been assumed that tissues damage occurs as a result of the spread of bacterial products and/or inflammatory mediators from the intestine to target organs (Griffin, 1995). In our experiment model, *E. coli* O157: H7 colonization was observed in the caecum and colon. Similarly, Nogano *et al*., (2003) reported effective intestinal colonization up to 28 days after oral infection with an *E. coli* strain isolated from a patient with HC, although no systemic symptoms or death were documented. Sperandio *et al*., (1999) suggested that intestinal colonization by *E. coli* O157: H7 could be induced by quorum sensing of signals produced by nonpathogenic *E. coli* of the normal intestinal microbiota. There are possibility for *E. coli* O157: H7 RIMD 05091078 and EDL 933 to show the colonization on caecum and colon of mice. Although *E. coli* O157: H7 were detected in the caecum and colon up to ten days after the inoculation, colonies of the pathogens were found on the caecum and colon but not on those of the small intestine. In this study, we demonstrated that Verocytotoxins are an important factor. Renal injury in infected mice would indicate that the toxin has reached systemic circulation. The histopathological changes such as enlarged glomerulus and endothelial cells damage in the infected mice were markedly more severe than those in the treated mice.

5. Effect of semi-purified Qi 4 fraction on enterohaemorrhagic *E. coli* O157: H7 in mice

This is the first report to describe the antibacterial activity of semi-purified Qi 4 fraction from nut galls against *E. coli* O157: H7. Based on these promising *in vitro* and *in vivo* assay findings, we believe that semi-purified Qi 4 fraction is likely to become a novel antimicrobial treatment for *E. coli* O157: H7. In

this study, we evaluated the effect of semi-purified Qi 4 fraction when administered to mice after oral bacterial inoculation. The semi-purified Qi 4 fraction at 1 mg/ml completely protected colonization and pathological changes in mice when administered 24 h after EHEC infection. The numbers of bacterial from faeces correlated with the number of colonized bacteria in the gastrointestinal tract. On day five, no organisms were detected in mice given semi-purified Qi 4 fraction at 4MIC (1 mg/ml). Semi-purified Qi 4 fraction from nut galls is hydrolyzable tannins which is known to have antibacterial activity against *S. aureus*, *Salmonella*, and *E. coli*. This tannin is highly water soluble and relatively rigid and spherical (Taguri *et al.*, 2004). Inhibition of *E. coli* O157: H7 correlated with highest ellagic acid concentration and /or antioxidant activity (Puupponen-Pimia *et al.*, 2005). The two suggested mechanisms of action were disruption of the cytoplasmic membrane by localized hyper-acidification, and disruption of membrane-transport and /or electron transport. These results suggest that semi-purified Qi 4 fraction can prevent intestinal colonization of *E. coli* O157: H7 organisms and histopathological changes of glomerulus. Semi-purified Qi 4 fraction from nut galls effectively protects mice against *E. coli* O157: H7 infection by preventing of colonization of the gastrointestinal tract and renal injury and colonization in mice.

CHAPTER 5

CONCLUSIONS

This final chapter of the thesis concludes the findings of the research study. It attempts to integrate the conclusions drawn from each experiment and identify future research needs. Finally, some general conclusion are drawn from the work.

1. Ethanolic extract and semi-purified Qi 4 fraction of nut galls were effective against *E. coli* O157: H7 RIMD 05091078, RIMD 05091083, EDL 933, O26: H11 RIMD 05091055, O111: NM RIMD 05091056, O22 RIMD 05091556, and *E. coli* ATCC 25922.

2. The semi-purified Qi 4 fraction of nut galls possess good antibactericidal activity against *E. coli* O157: H7. In contrast to many antibiotics, the effective semi-purified Qi 4 fraction inhibited the production of VT both in the periplasmic space (VT1) and the cell supernatant (VT2) at subinhibitory concentrations. The findings described here indicates that nut galls can effectively prevent both the growth of *E. coli* O157: H7 and the production of VT. Whether or not the presence of VT in food is a problem needs more investigation. However, since both VT1 and VT2 can result in serious complications, it would be of great advantage to find some safe and effective food additive which could inhibit both the growth of the organisms and the release of the toxins.

3. The ethanolic extract and semi-purified Qi 4 fraction of nut galls modulates the first mucosa contact surface phase of infection by enhancing cell surface hydrophobicity. Further studies are required to evaluate the biological effects of nut galls extract in conditions similar to those found *in vivo*. The information may contribute to the understanding of EHEC attachment to host intestinal mucosal surfaces and assist in the implementation of alternative treatment and prophylactic controls of these pathogens in modern medicine.

4. The 50% ethanolic extract and semi-purified Qi 4 fraction of nut galls have been shown to possess bacteriostatic and bactericidal properties on *E. coli* O157: H7. Electron micrographs of bacterial cells grown in the presence of minimal inhibitory concentration (MICs) showed disruption of the cell wall and the cytoplasmic membranes at the polar regions of the cells, loss of the double membranes structure, and some vacuolizations. The 50% ethanolic extract of nut galls completely killed the bacteria within 12 h at 4MIC (3.12 mg/ml). Bacterial cells also demonstrated distortions and collapse of the cell membranes, and fragmentation of the bacteria. The bacterial cells treated with semi-purified Qi 4 fraction at MIC (250 μg/ml) revealed blebs on cell surface and showing detachment of the cytoplasmoic membrane from the cell wall. These findings demonstrate that morphological and ultrastructural changes occurred when susceptible organisms are exposed to ethanolic extract and semi-purified Qi 4 fraction of nut galls.

5. The small intestine, caecum, and colon of mice colonized with *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933 appeared normal by light and electron microscopy.

6. Renal injury of IRC mice was induced by *E. coli* O157: H7 RIMD 05091078 and *E. coli* O157: H7 EDL 933. The glomeruli showed widening of mesangial cells, fragmentation of endothelial cells and hyperthophy.

7. The semi-purified Qi 4 fraction at 1 mg/ml effectively blocked the colonization of the organism in the intestinal tract and protected against renal injury of IRC mice.

Future research needs

Its is clear from the above that there are still may questions to be answered. Some specific suggestions for further research are listed as follows.

1. Establishing the effect of semi-purified Qi 4 fraction on the adherence by *E. coli* O157: H7 to cell cultures may identify aspects of the colonization process.

2. Evaluation of the effect of the semi-purified Qi 4 fraction on the normal intestinal microbiota in mice.

3. Further purification and attempts to determine the precise structure of the semi-purified Qi 4 fraction.

4. Evaluation of the prophylactic effect of the semi-purified Qi 4 fraction on *E. coli* O157: H7 infection in mice.

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APPENDICES

APPENDIX A

Culture Media

1. Mueller-Hinton Agar (MHA)

Beef extract	300	g
Casamino acids technical	17.5	g
Starch	1.5	g
Agar	15	g
Distilled water	1000	ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121 ^{o}C (15 psi) for 15 min.

2. Mueller-Hinton Broth (MHB)

Beef extract	300	g
Bacto Casamino acids technical	17.5	g
Bacto soluble starch	1.5	g
Distilled water	1000	ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121° C (15 psi) for 15 min.

3. Sorbitol MacConkey agar (SMAC)

Peptone	20	g
Sodium chloride	5	g
Bile salts no.3	31.5	g
Sorbitaol	10	g
Neutral red	0.03	g
Crystal violet	0.001	g
Agar	15	g

Dissolve 25.8 g in 500 ml distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121 ^{o}C (15 psi) for 15 min.

4. TSA (Tryptic Soy Agar)

Trypticase peptone	17	g
Phytone peptone	3	g
NaCl	5	g
Agar	15	g
Distilled water	1000	ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121° C (15 psi) for 15 min.

5. TSA (Tryptic Soy Broth)

Trypticase peptone	17	g
Phytone peptone	3	g
NaCl	5	g
K_2HPO_4	2.5	g
Dextrose	2.5	g
Distilled water	1000	ml

Dissolve the mixture by distilled water and boil until it was dissolved well and then sterilized by using an autoclave at 121 ^{o}C (15 psi) for 15 min.

APPENDIX B

Fixative and Staining

1. Neutral buffered 10% formalin for routine histology.

Tap water	900	ml
Formalin (37% formaldehyde solution)	100	ml
Sodium phosphate, monobasic, monohydrate	4	g
Sodium phosphate, dibasic, anhydrous	6.5	g

The pH should be 7.2-7.4

2. 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3

Paraformaldehyde, power	40	g
Sodium phosphate, monobasic	18.8	g
Sodium hydroxide (NaOH) solution (50% W/W)	4.3	ml
Distilled water	800	ml

Heat the above mixture at 60° C until it becomes a clear solution. Add distilled water to make 1000 ml, mix well. Cool to room temperature and adjust pH to 7.3 with 1N NaOH. Keep this fixative in refrigerator.

2. 3% Glutaraldehyde in phosphate buffer.

0.2 M phosphate buffer, pH 7.3

0.2 M sodium phosphate monobasic	23	ml
0.2 M sodium phosphate dibasic	77	ml

Mix and dilute to 200 ml to obtain pH 7.3. Take 6 ml of 50% (12 ml of 25% glutaraldehyde and make up to 100 ml with buffer. Store in cool, and use within 24 hours. Adding 10 drops of 1% CaCl per 100 ml of the fixative prior to use recommended. Time of fixation: 15 min to 12 h and followed by osmium fixation in the same buffer.

3. Stock 4% Osmic acid

Osmium tetroxide (crystal)	1	g
Distilled water	25	ml

Keep under the hood at room temperature.

Caution: Osmic acid vapor is highly toxic, therefore the preparation of the above solution must be done under the hood.

4. 2% Osmium tetroxide in 0.1 M phosphate buffer, pH 7.3.

4% Osmic acid	1	part
0.2 M phosphate buffer, pH 7.3	1	part

5. Toluidine blue stain for semi-thin sections

Sodium tetraborate (borax)	1	g
Toluidine blue	1	g
Distilled water	100	ml

Dissolve the borax in the distilled water and then add the toluidine blue. After filtering, the final solution can be stored at room temperature.

6. Uranyl acetate (2% aqueous)

Uranyl acetate	2	g
Distilled water	100	ml

Combine regents in proportions indicated. Filter, divide into suitable aliquots, and store at 4 ^{o}C in the dark. Centrifuge before use.

7. Reynolds' lead citrate stain (Reynolds 1963)

Stock reagents

Lead nitrate	2.66	g
Trisodium citrate	3.52	g
1M sodium hydroxide (freshly prepared)	16	ml
Distilled water (freshly prepared, carbonate-free)	84	ml

Mix the reagent in an alkaline-cleaned stoppered flask with approximately 60 ml of the water, inverting continuously for 1 min. Allow to stand for 30 min with occasional mixing. Add sodium hydroxide and mix until the solution becomes clear. Make up to 100 ml with remaining water. Divide into suitable aliquots and store at 4 ^oC. Centrifuge before use.

8. Stock 0.2 M phosphate buffer solutions.

Solution A

Sodium phosphate monobasic (NaH_2PO_4. H_2O)	27.6	g
Distilled water to make	1000	ml

Solution B

Sodium phosphate dibasic (Na_2HPO_4.)	28.4	g
Distilled water to make	1000	ml

9. 0.2 M phosphate buffer, pH 7.3

Stock Solution A (0.2 M NaH_2PO_4. H_2O)	23	ml
Stock Solution B (0.2 M Na_2HPO_4)	77	ml

Adjust pH to 7.3 and keep this buffer solution in refrigerator. To make 0.1M phosphate buffer, pH 7.3, is to dilute 0.2 M phosphate buffer with equal volume of distilled water.

APPENDIX C

Tissue Processing Schedules

1. TEM- Standard Tissue Processing

Step			
1. Primary fixation	2.5% glutaraldehyde in 0.1M phosphate buffer	2-24	h
2. Wash	0.1 M phosphate buffer	2X10	min on rotator
3. Post-fixation	1% aqueous osmium tetroxide	60	min
4. Wash	Distilled water	2X10	min
5. En-bloc staining	2% aqueous uranyl acetate	20	min
6. Dehydration	70% ethanol	10	min on rotator
	80% ethanol	10	min on rotator
	90% ethanol	10	min on rotator
	100% ethanol	15	min on rotator
	Dry absolute ethanol	2X15	min on rotator
7. Transition solvent (clearing)	propylene oxide	2X15	min on rotator
8. Infiltration	50:50, clearant: resin (Epon-812)	1 h	
	25:75, clearant: resin	1 h	
	Resin only	1-24 h	
9. Embedding	Fresh resin in embedding capsules	12-24 h at 60-70 ^{o}C	
10. Sectioning	Thick sections, 0.5-1 µm		
	Thin sections, 60-90 nm		
11. Staining	5% Uranyl acetate and Lead citrate		
12. Observation	TEM		

2. SEM- Standard Tissue Processing

Tissue, Cell suspensions on to Nucleopore or slides

1.	Primary fixation	2.5% glutaraldehyde in 0.1M phosphate buffer	2	h
2.	Wash	0.1 M phosphate buffer	2X15	min
3.	Post-fixation	1% aqueous osmium tetroxide	60	min
4.	Wash	Distilled water	2X15	min
5.	Dehydration	50% ethanol	15	min
		70% ethanol	15	min
		80% ethanol	15	min
		90% ethanol	15	min
		100% ethanol	2X15	min

6. Critical point dry as per the instruction manual
7. Orientate and stick specimens onto stubs using silver glue or double sided adhesive tape
8. Sputter coat with gold to a thickness of 15-20 nm as per the instruction manual
9. Store in a desiccator when not being viewed

3. Routine histological method

Tissue size	1X1X0.5 cm	
1. Fixation	10% formalin	12 h
2. Automatic tissue processing		
2.1 Dehydration	70%, 95%, 100% ethanol	7 h
2.2 Clearing	Xylene	2X3 h
2.3 Infiltration	Melted paraffin	6 h
3. Embedding in paraffin	Blocking	
4. Sectioning	Microtome knives, thick and thin sections, 5-6 μm	
5. Section adhesives	Slides	
6. Staining	Hematoxylin and Eosin stains	

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