Eleuthereine americana: A candidate for the control of Campylobacter species

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ABSTRACT The antibacterial activity of ethanolic extracts of selected Thai medicinal plants (Rhodomyrtus tomentosa (Aiton) Hassk., Quercus infectoria G. Olivier, and Eleutherine americana Merr.) against Campylobacter spp. was investigated. Sixty-five Campylobacter, including 39 isolates from humans and 26 isolates from chicken samples, were tested. Reference Campylobacter spp. that are commonly encountered in gastroenteritis were included. The ethanolic extract of E. americana demonstrated good antibacterial activity against all the tested isolates. Inhibition zones ranged from 10 to 37 mm. Minimum inhibitory concentration (MIC) of the extract against Campylobacter isolates from humans and chicken samples ranged from 31.25 to 500 μg/mL and 62.50 to 1,000 μg/mL, respectively. The minimum bactericidal concentration ranged from 31.25 to 1,000 μg/mL for isolates from humans and 125 to 1,000 μg/mL from chicken isolates. The bactericidal activity of the ethanolic extracts of E. americana against important Campylobacter spp., including Campylobacter coli MUMT 18630, Campylobacter fetus ATCC 27374, Campylobacter jejuni ATCC 81176, Campylobacter lari ATCC 43675, and Campylobacter upsaliensis DMST 19055, were assessed at MIC, 2 MIC, and 4 MIC by counting viable cells after various time intervals. At 4 MIC, the level of the tested isolates decreased by 2 to 5 log-fold within 8 h. The ethanolic extract of E. americana demonstrated antibacterial activity against all Campylobacter spp. from both human and chicken isolates. Further investigation of this plant species may provide an alternative medicine for Campylobacter infection and an effective food additive to prevent the infection.

Key words: Campylobacter spp., Thai medicinal plant, Eleutherine americana, antibacterial activity, time-kill study

INTRODUCTION

Foodborne diseases caused by Campylobacter spp. have recently emerged as a major public health issue in many places around the world. Eighteen species of the genus Campylobacter (Humphrey et al., 2007), particularly Campylobacter jejuni and Campylobacter coli, have emerged as major causes of human gastroenteritis. In sheep and cattle, some strains of C. jejuni and Campylobacter fetus can cause infertility and abortions (Institute for International Cooperation in Animal Biologies, 2005). Campylobacter fetus has been isolated from humans with septicemia. Campylobacter lari can cause disease but seems to be of minor importance to humans (Werno et al., 2002). In humans infected with the human immunodeficiency virus, Campylobacter upsaliensis has been detected and associated with gastroenteritis (Jenkin and Tee, 1998; Jimenez et al., 1999). In addition, Campylobacter can cause severe postinfection complications, including Guillain-Barré syndrome and reactive arthritis (Adedayo and Kirkpatrick, 2008).

Poultry is a known reservoir of Campylobacter spp. and play an important role in the transmission of Campylobacter enteritis to humans (Humphrey et al., 2007). In 2008, a total of 5,825 laboratory-confirmed cases of Campylobacter infection in the Foodborne Diseases Active Surveillance Network (FoodNet) observation areas were reported by the Centers for Disease Control and Prevention (CDC, 2009). In 2009, a total of 6,033 laboratory-confirmed cases of bacterial gastroenteritis reported by the Centers for Disease Control and Prevention were caused by Campylobacter (CDC, 2010). In 2010, the US Food and Drug Administration reported 12 cases of illness from Campylobacter infections in consumers who drank raw milk (FDA, 2010). New Zealand has the highest rate of Campylobacter infection in the world. These infections are attributable to multiple sources, including the consumption of raw or undercooked poultry (Campylobacter Attorney, 2009).

For the clinical treatment of Campylobacter spp. infections, erythromycin and quinolones are the most
frequently used antibiotics (Nachamkin et al., 2008). There have been many reports of *Campylobacter* resistance to antibiotics (Gibreel and Taylor, 2006; Payot et al., 2006; Miflin et al., 2007; Senok et al., 2007; Adekunle et al., 2009; Bostan et al., 2009; Gu et al., 2009). It has been claimed that the antimicrobials used in food animal production contribute significantly to the development of foodborne pathogens with resistance to antimicrobial agents (Boonmar et al., 2005; Quinn et al., 2007). Veterinary licensing of enrofloxacin in poultry was authorized by the European Union in 1991. In 1999, the European Union suggested that the use of fluoroquinolones in poultry be limited (Gallay et al., 2007). However, antibiotic resistance of *Campylobacter* isolates in humans and animals has increased remarkably in developing countries (Shapiro et al., 2001; Isenbarger et al., 2002). The development of fluoroquinolone-resistant *Campylobacter* strains after the introduction of these drugs for the treatment of infections in poultry has increased in broiler chickens (Savasan et al., 2004; Yıldırım et al., 2005).

Recently, medicinal plants are becoming more widely used on a commercial scale in the food industry, mainly for their actions as preservative agents. A wide range of medicinal plants have been demonstrated for their antagonistic effects against pathogenic bacteria (Voravuthikunchai et al., 2004, 2007; Voravuthikunchai and Kitpipit, 2005; Voravuthikunchai and Mitchell, 2008; Limsuwan et al., 2009a,b). In a previous study, an ethanolic extract of *Acacia nilotica* (Solomon-Wisdom and Shittu, 2010), aqueous leek extracts (Lee et al., 2004), crude extracts from *Drypetes gosseweileri* and *Pleiochrophorus* spp. (Tan et al., 2006), and a crude ethanolic leaf extract of *A. nilotica* (Raji et al., 2002) were reported to exhibit antibacterial effects against *Campylobacter* spp. The 3 most effective plants from our ongoing research work, namely, *Rhodomyrtus tomentosa*, *Quercus infectoria*, and *Eleutherine Americana*, were selected to assess their antibacterial activity against important *Campylobacter* spp. Different isolates from clinical specimens and chicken samples were studied for comparison purposes. This preliminary work demonstrates the potential use of extracts from *E. americana* as an anti-*Campylobacter* agent.

**MATERIALS AND METHODS**

**Preparation of Crude Extracts**

Leaves of *R. tomentosa*, nut galls of *Q. infectoria*, and bulbs of *E. americana* were collected and washed with distilled water. Classified reference voucher specimens were deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Samples were dried and then ground into powder. The powders were extracted with 95% ethanol at room temperature for 7 d. The extracts were evaporated using a rotary evaporator (Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland) until the extracts became completely dry and then were stored at 4°C. All extracts were dissolved in 10% dimethylsulfoxide (Sigma-Aldrich, Seelze, Germany) before use.

**Bacterial Isolates**

Thirty-nine human clinical isolates of *Campylobacter* spp. and 26 isolates from chicken samples were used in this study. Six reference strains, namely, *C. jejuni* ATCC 33291, *C. lari* ATCC 43675, *C. fetus* ATCC 27374, *C. upsaliensis* DMST 19055, *C. jejuni* ATCC 33650, and *C. jejuni* ATCC 81176, were used.

**Antibiotic Resistance Patterns**

Clinical isolates and isolates from chicken samples were checked for their antibiotic resistance profiles with selected antibiotics commonly used by the paper disk agar diffusion method (Clinical and Laboratory Standards Institute, 2006b) on Mueller-Hinton agar (MHA, Difco, Becton Dickinson, Sparks, MD) supplemented with 5% blood. The antibiotic disks (Oxoid) included ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), nalidixic acid (30 μg), streptomycin (10 μg), and tetracycline (30 μg). The experiments were performed in duplicate, and inhibition zones were averaged and compared with the standard values to categorize whether the antibiotic was susceptible or resistant.

**Antibacterial Activity of Crude Extracts on Campylobacter spp.**

The paper disc agar diffusion method was used. Ten microliters of crude extracts (250 mg/mL) dissolved in dimethylsulfoxide were added to a sterile filter paper disc (Macherey-Nagel GmbH, Düren, Germany). The discs were dried at room temperature overnight and applied on the surface of MHA supplemented with 5% blood seeded with broth culture of the test isolates adjusted to 10⁶ cfu/mL. The plates were then incubated at 37°C for 48 h under microaerophilic conditions. The experiments were performed in duplicate and the means of inhibition zone diameters were calculated.

**Determination of Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations**

A modified microdilution method (Clinical and Laboratory Standards Institute, 2006a) was used to determine the minimal inhibitory concentration (MIC) of extracts from *R. tomentosa*, *Q. infectoria*, and *E. americana*. The extracts (20 μL) were diluted to final concentrations ranging from 0.12 to 1.0 mg/mL in a 96-
well microtiter plate and 80 μL of Mueller-Hinton broth (Difco, Franklin Lakes, NJ). One hundred microliters of bacterial suspension (10⁶ cfu/mL) were inoculated and incubated at 37°C for 48 h under microaerophilic conditions. Controls with 1% dimethyl sulfoxide and without the extract were set up under the same conditions. Minimum inhibitory concentrations were observed at least in duplicate as the lowest concentration of plant extracts that produced a complete suppression of colony growth. Minimum bactericidal concentrations (MBC) were determined by streaking the contents of the microtiter wells that gave significant MIC on fresh MHA supplemented with 5% blood and incubating at 37°C for 48 h under microaerophilic conditions. The concentration at which no bacterial growth was visible after 48 h of incubation was regarded as the MBC.

**Time-Kill Assay**

The bactericidal activity of the extract was studied using a time-kill assay. The bacterial culture (10⁶ cfu/mL) was added to Mueller-Hinton broth supplemented with 5% blood containing the extract at MIC, 2 MIC, and 4 MIC, respectively. The tubes were incubated at 37°C under microaerophilic conditions. The samples were collected every 2 h for 24 h, and a control tube without the extract was incubated under the same conditions. The surviving bacteria were cultured on MHA supplemented with 5% blood.

**RESULTS AND DISCUSSION**

Measurements of the antibacterial activity of antibiotics on *Campylobacter* spp. isolated from 39 humans and 26 chicken samples by the disc diffusion method were carried out (Table 1). The patterns between the 2 groups were very similar. About 26, 64, 5, 84, 3, and 30% of the isolates from humans with campylobacteriosis and 73, 77, 8, 84, 46, and 38% of the isolates from chicken samples were resistant to ampicillin, ciprofloxacin, erythromycin, nalidixic acid, streptomycin, and tetracycline, respectively.

Although 100% of the isolates were still susceptible to chloramphenicol and gentamicin, a relatively high proportion demonstrated resistance to the other antibiotics tested. Previous studies in Thailand showed the emergence of *Campylobacter* spp. resistant to a variety of antimicrobial agents, including nalidixic acid and ciprofloxacin (Hoge et al., 1998; Murphy et al., 1995; Padungtod et al., 2003).

Preliminary evaluations of the antibacterial activity of *Rhodomyrtus tomentosa*, *Quercus infectoria*, and *Eleutherine americana* ethanolic extracts were performed by the paper disc agar diffusion assay. The antibacterial effects of *R. tomentosa* and *Q. infectoria* on *Campylobacter* spp. were mild. The extract from *R. tomentosa* produced 36 and 16% inhibition against the human and chicken sample isolates, respectively. The results showed that the extract from *Q. infectoria* inhibited about 46% of human isolates.

### Table 1. Antibiotic resistance profiles of *Campylobacter* spp. isolated from humans (n = 39) and chicken samples (n = 26)

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Humans</th>
<th>Chicken samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (%)</td>
<td>I (%)</td>
</tr>
<tr>
<td>Ampicillin (10 μg)</td>
<td>27 (69)</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Chloramphenicol (30 μg)</td>
<td>39 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ciprofloxacin (5 μg)</td>
<td>11 (28)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Erythromycin (15 μg)</td>
<td>37 (95)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gentamicin (10 μg)</td>
<td>39 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nalidixic acid (30 μg)</td>
<td>6 (15)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Streptomycin (10 μg)</td>
<td>38 (97)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tetracycline (30 μg)</td>
<td>26 (67)</td>
<td>1 (3)</td>
</tr>
</tbody>
</table>

1S = susceptible; I = intermediate; R = resistant. Number of isolates (% of isolates in parentheses).

### Table 2. Antibacterial activity of ethanolic extracts (2.5 mg/disc) from *Rhodomyrtus tomentosa*, *Quercus infectoria*, and *Eleutherine americana* on *Campylobacter* spp. isolated from humans (n = 39) and chicken samples (n = 26) by the disc diffusion method

<table>
<thead>
<tr>
<th>Inhibition zone (mm)</th>
<th><em>R. tomentosa</em></th>
<th><em>Q. infectoria</em></th>
<th><em>E. americana</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Humans (%)</td>
<td>Chickens (%)</td>
<td>Humans (%)</td>
</tr>
<tr>
<td></td>
<td>Chickens (%)</td>
<td>Humans (%)</td>
<td>Chickens (%)</td>
</tr>
<tr>
<td>No inhibition zone</td>
<td>25 (64)</td>
<td>22 (84)</td>
<td>21 (54)</td>
</tr>
<tr>
<td>10</td>
<td>3 (8)</td>
<td>0 (0)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>15</td>
<td>3 (8)</td>
<td>1 (4)</td>
<td>9 (23)</td>
</tr>
<tr>
<td>20</td>
<td>5 (12)</td>
<td>1 (4)</td>
<td>5 (12)</td>
</tr>
<tr>
<td>25</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td>5 (12)</td>
</tr>
<tr>
<td>30</td>
<td>2 (5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>35</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>40</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

1Number of isolates (% of isolates in parentheses).
and 16% of the chicken sample isolates. In contrast, the ethanolic extract of *E. americana* demonstrated good antibacterial properties against all the tested isolates. The inhibition zones of the isolates were from 10 to 37 mm. Most isolates, from both the human and chicken samples, produced inhibition zones between 26 and 30 mm (Table 2). The MIC and MBC of the ethanolic extract from *E. americana* against *Campylobacter* isolates are presented in Table 3. The MIC values of the extract against *Campylobacter* isolates from humans and chicken samples ranged from 31.25 to 500 μg/mL and 62.5 to 1,000 μg/mL, respectively. The MIC50

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Humans</th>
<th>Chickens</th>
<th>MBC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC50</td>
<td>MIC90</td>
</tr>
<tr>
<td><em>E. americana</em> extract</td>
<td>31.25–500</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1–4</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

**Figure 1.** Effect of the ethanolic extract of *Eleutherine americana* on the survival of *Campylobacter coli* MUMT 18630 (A), *Campylobacter fetus* ATCC 27374 (B), *Campylobacter jejuni* ATCC 81176 (C), *Campylobacter lari* ATCC 43675 (D), and *Campylobacter upsaliensis* DMST 19055 (E). The viable cells in the control suspension (○) and after treatment with the extract at the minimum inhibitory concentration (MIC; ■), 2 MIC (▲), and 4 MIC (♦) were determined. The lower detection threshold was 10^2 cfu/mL.
(i.e., 50% MIC) and MIC90 (i.e., 90% MIC) values of the isolates from humans were 125 and 250 μg/mL, respectively. Similarly, the MIC50 and MIC90 values of the isolates from chicken samples were 125 and 50 μg/mL, respectively. In a previous study, crude extracts from D. gosseweileri and Pleiocarpa spp. were reported to exhibit an antibacterial effect against C. jejuni and C. coli, with the lowest MIC being 0.78 and 1.56 μg/mL, respectively (Tan et al., 2006). Lee et al. (2004) reported that the minimum concentration of aqueous leek extracts required to inhibit the bacterial growth was 2.0 mg/mL. Our results clearly indicated that the ethanolic extract from E. americana was comparatively effective against Campylobacter spp. The MBC values of the extract on Campylobacter isolates from humans and chicken samples ranged from 31.25 to 1,000 μg/mL and 125 to 1,000 μg/mL, respectively.

Series of studies on the antibacterial activity of E. americana against S. aureus have been documented (Iesan and Voravuthikunchai, 2009; Iesan et al., 2009a,b,c,d,e). Phytochemical studies on the bulb of E. americana revealed various types of naphthoquinones and anthraquinones (Komura et al., 1983; Zhengxiong et al., 1986; Hara et al., 1997; Xu et al., 2006). Some of these compounds were also found to possess diverse biological activities, such as being inhibitory against the human immunodeficiency virus, being inhibitory against topoisomerase II, and having antifungal activity (Zhengxiong et al., 1986; Xu et al., 2006).

The bactericidal activity of the ethanolic extract of E. americana on Campylobacter strains was assessed at MIC, 2 MIC, and 4 MIC by counting viable cells after time intervals. At 4 MIC, the levels of the tested isolates decreased by 2 to 5 log-fold within 8 h (Figure 1). At 4 MIC, the numbers of viable cells of C. coli MUMT 18630 (Figure 1A), C. jejuni ATCC 81176 (Figure 1C), C. lari ATCC 43675 (Figure 1D), and C. upsaliensis DMST 19055 (Figure 1E) were reduced by 2 to 3 log-fold within 8 h, except C. fetus ATCC 27374 (Figure 1B). However, within 18 h after being treated with the extract, both at 2 MIC and 4 MIC, the cell numbers of all species of Campylobacter decreased to levels at limit of detection. Patterns of cell survival after treatment were similar among isolates from humans and chicken samples. The ethanolic extract of E. americana demonstrated antibacterial activity against Campylobacter spp. Further investigation of this plant species may provide an alternative medicine for Campylobacter infection that could also be applied as a food additive to prevent the infection.

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