Short Communication

Antimicrobial Activity of Commercial Citrus-Based NaturalExtracts Against *Escherichia coli* O157:H7 Isolates and Mutant Strains

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Abstract

Due to increasing concerns about the development of antimicrobial resistance amongst pathogenic bacteria, alternative strategies have been sought that do not use antibiotics to reduce pathogenic bacteria from foods and patients. A natural compound that has potent antimicrobial properties is citrus peel, which contains a variety of essential oils that inhibit the growth of or kill pathogenic bacteria. In the present study, seven citrus-based natural antimicrobials were evaluated for their ability to inhibit the growth of the pathogen *Escherichia coli* O157:H7. Zones of inhibition of *E. coli* O157:H7 by the citrus-derived fraction (10 μL/6 mm disk) were determined by a disk-diffusion assay on Sorbitol-MacConkey agar. Inhibition zones were observed after 48 h lawn growth of *E. coli* O157:H7 cells at 37°C. Two citrus-based fractions, orange CP VAL terpeneless FAB 968611 and Limonene 1× Dist FAB 955430, inhibited *E. coli* O157:H7 with inhibition zones of approx. 11–24 mm dia. The remaining other five citrus-derived extracts (orange oil FL VAL 1121 ARR 974760, Orange 5× Conc VAL 4121 ARR 968374, orange terpenes ESS 1120 ARR 986259, orange terpenes CP 1100 ARR 986255, and orange terpenes OEO HP 1100 ARR 986257) were noninhibitory to *E. coli* O157:H7, yielding no clear inhibition zones. These studies show that citrus-derived natural compounds differ in their inhibitory activity against *E. coli* O157:H7 and some have potential applications as inhibitory agents against *E. coli* O157:H7 in various pathogen reduction strategies.

Introduction

Foodborne pathogen contamination is a major concern for the food processing industry, food producers, regulatory agencies, and the consumers. Enterohemorrhagic *Escherichia coli* (EHEC; including the well-known pathogenic strain O157:H7) has emerged as a major cause of foodborne illness and even death in the United States, causing as many as 90,000 human illnesses and 70 deaths per year in the United States at a cost of more than $1 billion USD (USDA-ERS, 2001). The primary route of infection with EHEC is consumption of undercooked...
or improperly cooked ground beef (Griffin, 1998; Jay et al., 2007). The dose of E. coli O157:H7 necessary to cause human illness has been estimated to be less than 1000 cells (Mead et al., 1999). Much of the human exposure through foods is due to the carriage of foodborne pathogenic bacteria in the intestinal tract of cattle and other ruminants, often as an undetectable resident organism (Low et al., 2005). EHEC can be introduced into the abattoir in the feces or attached to the hides of cattle (Reid et al., 2002; Aslam et al., 2003; Barkocy-Gallagher et al., 2003) and receiving a direct passage into the food chain on contaminated meats. E. coli O157:H7 have been carried to humans by both drinking and irrigation water (Anonymous, 2000), other animal or insect vectors (Ahmad et al., 2007; Jay et al., 2007), and direct animal contact (Chapman et al., 2000; Pritchard et al., 2000; Keen et al., 2007). Other foodstuffs that come into contact with ruminant animals or their manure can be contaminated with EHEC, and human illness outbreaks have been linked to such diverse sources as sprouts, lettuce, and apple cider (Besser et al., 1993; Itoh et al., 1998; Duffy et al., 2000; Jay et al., 2007).

Many plants contain compounds that demonstrate antimicrobial activity (Dorman et al., 2000) and can alter the microbial ecology of the surface of a food. (Hristov et al., 2001; Gill and Holley, 2006; Nam et al., 2006). Citrus products contain a wide variety of oils that are toxic to bacteria (Kim et al., 1995; Fisher and Phillips, 2006). Because of the need to develop new intervention strategies to reduce the risk of survival of very low numbers of E. coli O157:H7 that contaminate different food processing environments as well as in and on different food matrices, it has been proposed to use some of the oils found in citrus fruits to reduce pathogens in and on foods. Our preliminary work reported herein demonstrates differences in inhibitory activities of citrus-based antimicrobials against E. coli and E. coli O157:H7 in agar-disk diffusion model assays.

**Materials and Methods**

Seven citrus essential oils used in this study were obtained from Firmenich Citrus Center (Safety Harbor, FL) and were stored at 4°C per manufacturer’s recommendations prior to use. These orange oil fractions were designated with numbers C2 to C8, and physiochemical properties were described elsewhere (unpublished data). All these seven essential oils were evaluated for antimicrobial activities against 13 E. coli strains (Tables 1 and 2). The antimicrobial activity of the citrus oils was determined by an agar-disk diffusion assay as described by Woods and Washington (1995) and NCCLS (2002). Briefly, freshly grown E. coli or E. coli O157:H7 cells (approximately 10⁶ colony-forming units/mL) were surface spread-plated on Sorbitol-MacConkey agar and air-dried for 5 min. A sterile 6 mm filter disk was placed in the center of the Sorbitol-MacConkey agar plate, and 10 μL of undiluted citrus oil was transferred onto the filter disk. After being air-dried for 30 min, plates were incubated at 37°C for 48 h. Negative controls were disks soaked with

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**Table 1. Escherichia coli Strain and E. coli O157:H7 Isolates and Mutant Strains Used in This Study**

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Source ID</th>
<th>Characteristics/isolation source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E. coli</td>
<td>ATCC 11775</td>
<td>Human</td>
</tr>
<tr>
<td>2</td>
<td>E. coli O157:H7</td>
<td>301C</td>
<td>Chicken isolate</td>
</tr>
<tr>
<td>3</td>
<td>E. coli O157:H7</td>
<td>204P</td>
<td>Pork isolate</td>
</tr>
<tr>
<td>4</td>
<td>E. coli O157:H7</td>
<td>932</td>
<td>Human isolate</td>
</tr>
<tr>
<td>5</td>
<td>E. coli O157:H7</td>
<td>933</td>
<td>Beef isolate</td>
</tr>
<tr>
<td>6</td>
<td>E. coli O157:H7</td>
<td>C7929</td>
<td>Apple cider isolate</td>
</tr>
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<td>E. coli O157:H7</td>
<td>505B</td>
<td>Beef isolate</td>
</tr>
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<td>E. coli O157:H7</td>
<td>F501</td>
<td>CDC</td>
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<td>F585</td>
<td>CDC</td>
</tr>
<tr>
<td>10</td>
<td>E. coli O157:H7</td>
<td>ATCC 43895</td>
<td>SLT I &amp; II positive</td>
</tr>
<tr>
<td>11</td>
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<td>ATCC 43888</td>
<td>Hamburger isolate</td>
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<td>ATCC 43889</td>
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<td>ATCC 43890</td>
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<td></td>
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</tbody>
</table>
phosphate-buffered saline (pH 6.5). The zones of inhibition were measured after 48 h of incubation, and each citrus oil fraction was tested in three independent experiments.

**Statistical analysis**

Statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL). Mean differences were established by one-way analysis of variance (ANOVA) using general linear model procedure and Bonferroni adjustment for the multiple comparisons. Statistical differences among data were considered significant at \( p < 0.05 \).

**Results**

Of the seven citrus oil compounds tested in the present study, only two were inhibitory to *E. coli* and *E. coli* O157:H7 in agar-disk diffusion assay, and presented in Table 2. These two inhibitory citrus fractions were distilled hydrocarbon single fold d-limonene (C7) and Orange CP VAL Terpeneless FAB 968611 (C4). The other five citrus-derived extracts tested exhibited no zones of inhibition on plates of *E. coli* or *E. coli* O157:H7 (data not shown). Limonene was inhibitory to *E. coli* and to all 12 *E. coli* O157:H7 isolates tested, and yielded inhibition zones varying from 17.5 ± 0.7 mm to 37.5 ± 3.5 mm diameter. Compared to the C7 fraction, C4 was less inhibitory to all strains. The inhibition zones achieved with C4 were significantly smaller than the inhibition zones of the corresponding strains upon limonene (C7) application. Anti-microbial activities of both fractions, C4 and C7, appeared to be strain specific. The most susceptible to limonene (C7 fraction) of the *E. coli* strains were ATCC 43888 and O157:H7 932, which were not significantly different from *E. coli* strains ATCC 43890, ATCC 43895, and ATCC 11775. With inhibition zones of 14.5 ± 0.7 mm and 13.5 ± 0.7 mm, the *E. coli* O157:H7 ATCC 43890 and O157:H7 301C were the most sensitive strains to C4 fraction. All other *E. coli* O157:H7 strains yielded significantly smaller inhibition zones.

**Discussion**

Citrus oils are complex mixtures; approximately 400 compounds can be found in citrus fruits depending on the citrus cultivar, extraction, and separation methods (Braddock, 1999). Essential oils are an important component of the citrus fruit, and they act as natural antimicrobials (Kim et al., 1995; Fisher and Phillips, 2006). The essential oils from citrus products exert their bactericidal effects at the membrane level (Dusan et al., 2006; Di Pasqua et al., 2007), where they increase the permeability of the cell membrane.
(Gill and Holley, 2006). The best characterized of the essential oils from citrus products include citrullene and limonene, which can exert potent antimicrobial activity (Di Pasqua et al., 2006). The known antimicrobial activity of the essential oils has been shown to encompass E. coli strains (Dusan et al., 2006), some Salmonella spp. (Kim et al., 1995; Parish et al., 2003; Nam et al., 2006), and other foodborne pathogenic bacteria (Megias et al., 1997; Fisher and Phillips, 2006). In other studies examining the efficacy of orange oils to control pathogenic bacteria in and on foods, strains of Arcobacter butzleri and A. cryaerophilus spp. were only inhibited by the C4 fraction, while Campylobacter jejuni and C. coli were strongly inhibited by various citrus-based fractions, including cold pressed terpeneless Valencia orange oil (C4 fraction), fivefold concentrated Valencia orange oil, and distilled d-limonene (C7 fraction) (unpublished data).

When citrus oils are extracted, the two main fraction groups are the oxygenated compounds and the hydrocarbon fraction. Oxygenated compounds are polar, and they constitute less than 5% of the volume and contain the esters, aldehydes, ketones, alcohols, phenols, and oxides, giving citrus oils their characteristic fragrance and aroma that are used as fragrance and food ingredients. The hydrocarbon fraction is composed of nonpolar compounds that comprise more than 95% of most citrus oils. The principal hydrocarbon of citrus oil is d-limonene, which can be distilled to produce a nearly odorless compound. All of the hydrocarbons have low solubility in water, but can form emulsions with nonionic surfactants (NAID) so that they can be sprayed on the surfaces of meat, poultry, or vegetable products. Citrus oils are considered “Generally Recognized as Safe” by the U.S. Food and Drug Administration, partly based on the long history of consuming these oils naturally, and as flavoring agents in citrus juices.

The possibility of using essential oils from natural sources to inhibit pathogens is not a novel concept (Friedman et al., 2002; Dusan et al., 2006; Fisher and Phillips, 2006); however, the use of specific oil fractions to eliminate certain foodborne pathogens is a new technology and application that can be utilized to improve food safety. By defining the specific components in the oils that act to kill pathogens, we can better utilize the components that exhibit the best killing effect for specific pathogens in targeted environments. For example, the essential oil to be used on ground beef or luncheon meats would differ from the most appropriate one for use on lettuce, or to be used in animal feeds to reduce pathogens in the gut of the live animal. The erection of multiple, sequential barriers in the food production continuum will enhance the overall effectiveness of interventions by reducing total human exposures to pathogens, resulting in reduced total illnesses. Our study demonstrates that two of the seven citrus-derived natural compounds tested herein have potential applications as inhibitory agents against E. coli and E. coli O157:H7, and need further work on food matrices to determine their efficacy.

Acknowledgments

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References


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