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Grapefruit bioactive limonoids modulate *E. coli* O157:H7 TTSS and biofilm

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ABSTRACT

Limonoids are important constituents of the grapefruit and other citrus fruits. Research on health benefits suggests that citrus limonoids may act as anti-cancer, cholesterol lowering, anti-HIV and anti-feedant compounds. However, antimicrobial activities of citrus limonoids are not reported. In the present investigation, limonoids were purified from grapefruit seed and evaluated for their potential to antagonize cell-to-cell communication, biofilm formation and expression of Enterohemorrhagic *Escherichia coli* (EHEC) type three secretion system (TTSS). The results of the present study suggest that, certain limonoids are inhibitory to the cell-to-cell communication, biofilm formation and EHEC TTSS. Specifically, obacunone demonstrated strong inhibition of EHEC biofilm formation and TTSS. Furthermore, obacunone and other limonoids seem to inhibit the biofilm formation and TTSS in quorum sensing dependent fashion. The results indicate that certain grapefruit limonoids may possibly help in antagonizing the EHEC infection process, and may serve as lead compound in development of new antipathogenic molecules.

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1. Introduction

Enteric pathogen *Escherichia coli* O157:H7 (EHEC) is a shiga-toxin producing agent and causes wide spectrum of diseases including hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Rasmussen and Casey, 2001). Several food borne and water borne outbreaks have been reported throughout the world in the past including a multi-state outbreak in US in June 2009 (CDC Update June 30, 2009). The CDC estimates are about 73,000 cases of infection and 61 deaths caused by EHEC. The problem is further confounded by increased risk of hemolytic uremic syndrome and renal failure upon antibiotic treatment (Tarr et al., 2005; Wong et al., 2000).

EHEC colonizes large intestine and produces histopathological lesions known as attaching and effacing lesions (A/E lesions). In addition, EHEC produces potent shiga toxin, which is responsible for the major symptoms of HUS (Rasmussen and Casey, 2001). Formation of A/E lesions is facilitated by type III secretion system (TTSS) encoded in locus of enterocyte effacement (LEE) (McDaniel et al., 1995). Regulation of LEE is tightly controlled by several factors including autoinducer (AI)-2 and AI-3 mediated cell–cell signaling (quorum sensing) (Bansal et al., 2008; Sperandio et al., 2003). In addition, another pathogenic trait, biofilm formation in *E. coli* is regulated by complex interplay of environmental factors and cell signaling mediated by AI-2, AI-3, indole and Acyl-homoserine lactones (Gonzalez Barrios et al., 2006; Herzberg et al., 2006; Lee et al., 2007b). Similar to other *E. coli* strains, EHEC is known to form biofilms in various conditions (Lee et al., 2007a; Yoon and Sofos, 2008). In addition, production of shiga toxin is influenced by quorum sensing (Sperandio et al., 2001). Thus, autoinducer mediated cell–cell signaling serves as a central regulator of EHEC pathogenicity, and an interference with this signaling may curtail the EHEC virulence (Rasko et al., 2008). Strategies to effectively control EHEC infections and biofilm formation are urgently required since there are no adequate means available to combat these problems. Antagonizing quorum sensing is proposed as a novel mechanism with unconventional target to modulate pathogenicity (Rasmussen and Givskov, 2006).

Several phytochemicals are reported to interfere with cell–cell signaling (Huber et al., 2003; Manefield et al., 2002; Persson et al., 2005) and biofilm formation (Huber et al., 2003; Ren et al., 2001; Ren et al., 2005). Citrus fruits are important part of diet and contain a unique group of secondary metabolites known as limonoids. Limonin, nomilin, obacunone, deacetyl nomilin and limonin 17-O-β-D-glucopyranoside (Fig. 1) are the predominant limonoids present in the grapefruit (*Citrus paradisi* Macfad.) seed and juice (Hasegawa et al., 1989; Rouseff and Nagy, 1982). Several citrus limonoids are under investigation primarily for their possible anti-cancer properties (Lam and Hasegawa, 1989; Poulose et al., 2005; Poulose et al., 2006; Vanamala et al., 2006). Citrus limonoids are characterized by high degree of oxygenation and the presence of a furan ring. In comparison,

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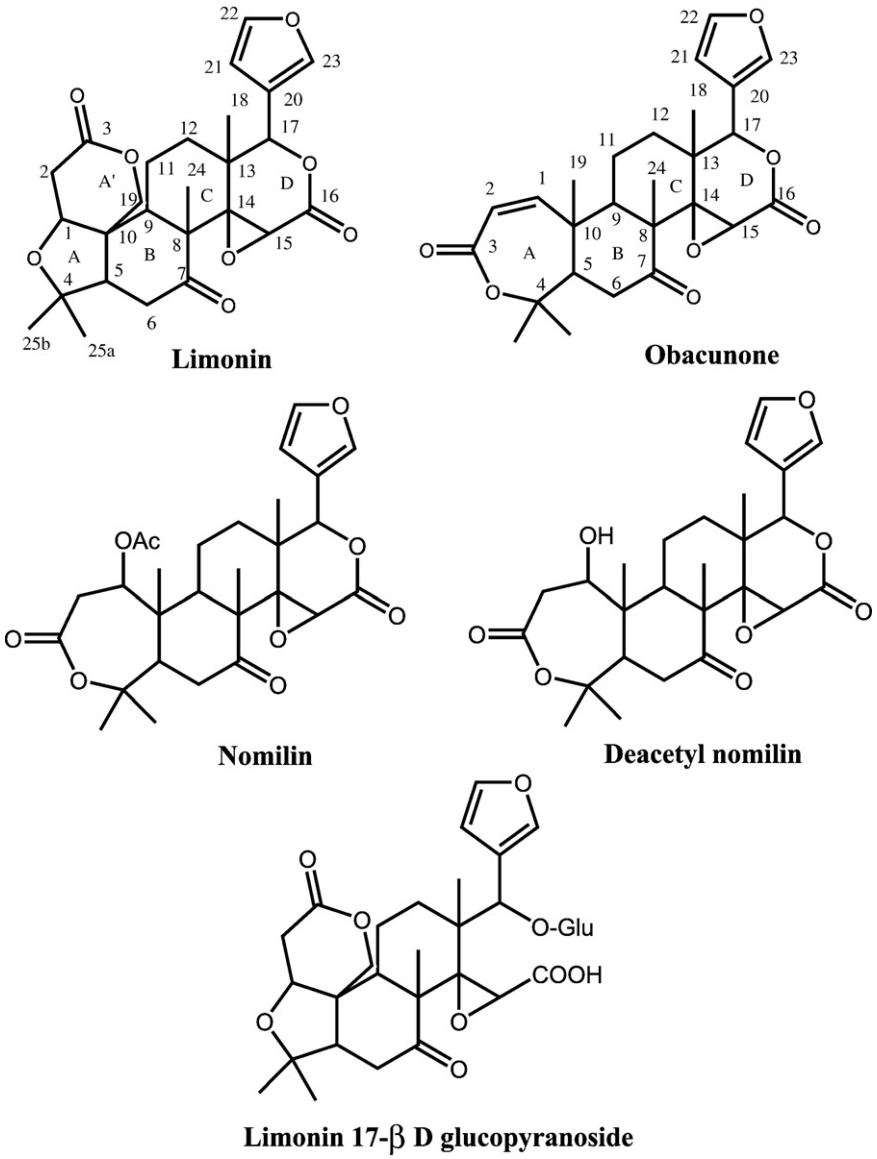

Fig. 1. Structures of limonoids (A. Limonin, B. Obacunone, C. Nomilin, D. Deacetyl Nomilin, E. Limonin 17-β-D-glucopyranoside).

autoinducer molecules HAI-1, AI-2 are furanones. These observations led us to investigate antagonistic activities of grapefruit limonoids against quorum sensing and regulated processes such as bioluminescence and biofilm formation. In the current study, we present the evidence that grapefruit limonoids inhibit cell–cell communication, EHEC biofilm formation and more importantly attenuate EHEC TTSS.

2. Materials and methods

2.1. Materials

Acetone, methanol, ethyl acetate, acetonitrile (Reagent and HPLC grade) and thin layer chromatographic silica-gel 60F-254 plates were purchased from Fischer Scientific (Hampton, NH). S-adenosylhomocysteine was purchased from Sigma-Aldrich (St. Louis, Mo., USA).

2.2. Extraction and purification of limonoids

Grapefruit (*C. paradisi*) seeds were collected from Texas A&M University, Kingsville, Citrus Center, Weslaco, Texas, USA. The seeds were separated from the fruits and air-dried under shade at 25 °C for 7–8 days to obtain ≈2% moisture level and ground to powder (40–60 mesh size).

Defatted seed powder (2.0 kg) was extracted successively for 8 h each with acetone and methanol:water in a soxhlet apparatus. The limonoids were purified according to previously published methods from our laboratory (Mandadi et al., 2007). In brief, the extracts were filtered and concentrated under vacuum (Buchi, Switzerland). The acetone fraction (42 g) was partitioned between dichloromethane (DCM) and water (2:1). Compound **1** crystallized in the DCM. *In vacuo* dried supernatant (27 g) was chromatographed on silica-gel (300 g) column (3.5×90 cm) and eluted with linear gradient of 1% ethyl acetate (EtOAc) in DCM. Compounds **2**, **1**, **3** and **4** were eluted with DCM/EtOAc (99:1), (95:5), (97.5: 2.5), (80:20) respectively.

The methanol extract was purified according to Jayaprakasha et al. (2007). Briefly, methanol extract was loaded onto an activated dowex [H^+] resin column and washed with deionized water. Elute from dowex column was passed through a sepabeads resin column, which was eluted with a step-wise gradient of 2.5% acetonitrile in water. The fractions depicting similar profile were pooled and concentrated under