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Antimicrobial and immunomodulating activities of hesperidin and ellagic acid against diarrheic *Aeromonas hydrophila* in a murine model

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ABSTRACT

Aims: The present study is designed to evaluate the in vitro and in vivo bactericidal and immunomodulating activities of hesperidin (HES) and ellagic acid (EA) against *Aeromonas hydrophila*. *A hydrophila*, an uncommon human pathogen, can cause invasive infections in immunocompromised individuals and common clinical presentations in acute gastrointestinal illness, soft-tissue infections and sepsis. The antimicrobial activities of medicinal plants against *A. hydrophila* have received only cursory attention.
Methods: We examined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in vitro. Moreover, the effects of HES and EA against bacterial colonization were studied in vivo. Also, humoral immune response was tested against *A. hydrophila*-LPS or *A. hydrophila*-ECP antigen preparations and the intestinal histopathological alterations were studied.
Results: Data revealed that the treatments with HES and EA each had antimicrobial activities against *A. hydrophila*. Both HES and EA treatments significantly increased anti-LPS IgM levels and reduced anti-LPS and anti-ECP IgA levels to their normal values in comparison to the infected group, which recorded significantly elevated levels two week post-infection. In conclusion, the present data suggest that HES and EA have antimicrobial and immunomodulating activities against murine *A. hydrophila* infections.
Significant: These data warrant clinical studies to delineate HES and EA roles in human infectious diseases.

Introduction

Aeromonas hydrophila is an emerging human pathogen that causes both intestinal and extraintestinal infections, including diarrhea, septicaemia, cellulitis, and other infections in wounds and soft tissue (Vila et al., 2003; Galindo et al., 2006). The ability of *A. hydrophila* to resist the effects of multiple antibiotics poses a potential threat in choosing therapeutic modalities (Khajanchi et al., 2011). *A. hydrophila* can invade the human body through contaminated wounds and, in 2012, it was associated with a severe case of necrotizing fasciitis in a young woman, resulting in the loss of one leg (USA-FDA, 2012).

It is well known that the extent of antibiotic resistance defines the epidemiology of infectious diseases and the pharmacological therapies that might be employed (Tenover, 2006). To avert the menace of pathogens resistant to antibacterial agents, research is now increasingly focused on discovering new antimicrobial compounds from both microbial and plant origins (Brehm-Stecher and Johnson, 2003; Røssland et al., 2005). Secondary metabolites derived from natural products include pharmacologically bioactive compounds such as alkaloids, flavanoids, tannins, anthraquinones and phenols (Edeoga et al., 2005; Nawrot et al., 2007). Hesperidin (HES) is a flavanone glycoside commonly found in the diet in citrus fruits or citrus fruit derived products. HES is known to inhibit inflammation, hypotension and analgesia, and nitric oxide synthase (Olszanecki et al., 2002). The anti-inflammatory effects of HES in vitro have been characterized in both rodent and human cell lines (Choi et al., 2003; Sakata et al., 2003). Yeh et al. (2007) have demonstrated that HES effectively mitigates LPS-induced acute lung injury. Bakar et al. (2012) have showed that HES exhibits antimicrobial activities against vancomycin-intermediate *Staphylococcus aureus* in vitro and against seven of the eight strains of Gram-positive and Gram-negative bacteria (Basile et al., 2000). Most recently, HES has been reported to exhibit pronounced immunological activities, serving to inhibit inflammatory cell infiltration and mucus hypersecretion in a murine model of asthma (Wei et al., 2011).

Ellagic acid (EA), meanwhile, is a dimeric derivative of gallic acid and a phenolic compound found in many fruits. Interest in EA has increased recently due to its potential antimutagenic and anticarcinogenic effects (Heber, 2008). EA is a potent antioxidant, acting as a scavenger of oxygen species produced by hydrogen peroxide treatment, and as a

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protector of the DNA double helix from alkylating agent injury. Moreover, EA has been recorded as having anti-microbial activity (Thiem and Goślińska, 2004; Choi et al., 2011).

While the properties and mode of action of both HES and EA are increasingly understood, therefore, their antimicrobial activities, especially against *A. hydrophila*, have received only cursory attention. Moreover, researchers have recently found an increasing resistance to broad-spectrum cephalosporins in clinical *Aeromonas* isolates and, since the optimal therapy for invasive infections caused by cefotaxime-resistant *A. hydrophila* is still unknown, the present study aimed to evaluate the bactericidal effects of hesperidin (HES) and ellagic acid (EA) on *A. hydrophila* through both in vitro and in vivo studies.

Materials and methods

Bacteria and growth conditions

A standard *A. hydrophila* strain (ATCC; Cat. #7966) was kindly provided by the Fish Department, Faculty of Veterinary, Cairo University, Giza, Egypt. The bacterium was maintained and subcultured three times before the experiments reported here. Briefly, 100 μl of *A. hydrophila* was inoculated into 150 ml of a liquid peptone broth (Oxoid) and incubated for 30 °C for 24 h with continuous shaking at 250 rpm. The harvested bacteria were centrifuged at 6000 *g* for 10 min and the dried pellet was suspended twice in phosphate-buffered saline (PBS) to the final count of dose of 2×10^8 CFU/ml.

Preparation A. hydrophila-lipopolysaccharides (A-LPS)

LPS was prepared as described by Westphal and Jann (1965). Briefly, the bacteria were inoculated in 250 ml of Luria–Bertain broth (LB) and incubated for 24 h at 30 °C on a shaker at 250 rpm. The culture was then centrifuged at 10,000 rpm for 10 min at 4 °C, resuspended in 16.6 ml of TAE buffer (40 mM Tris-acetate, pH 8.5; 2 mM EDTA) and then mixed with 33.2 ml alkaline solution (containing 3 g of SDS, 0.6 g of Trizma (Sigma), 160 ml of 2 N NaOH in 1000 ml of water). The suspension was heated at 55 to 60 °C for 70 min and then mixed with phenol and chloroform in the ratio of 1:1 (V/V). The mixture was spun at 10,000 rpm for 10 min at 4 °C and the supernatant obtained was mixed with 33.2 ml of water and 8.3 ml of 3 M sodium acetate buffer (pH 5.2). LPS was precipitated by adding twice the volume of ethanol. The precipitate was dissolved in 33.2 ml of 50 mM Tris–HCl, pH 8.0 (Sigma) and 100 mM sodium acetate, mixed well, and was then re-precipitated with twice the volume of ethanol. The combined water extract was dialyzed for 2–4 days against distilled water and then freeze dried.

Preparation A. hydrophila-extracellular proteins [A-ECP]

The bacterial isolate was grown overnight in 5 ml LB broth for preculturing. 100 μl of this culture suspension (inoculum) was added to 50 ml LB broth and incubated overnight at 37 °C at a shaker speed of 200 rpm. The culture suspension was harvested at 5000 rpm at 4 °C for 15 min. The supernatant was precipitated by the addition of 10% (w/v) trichloroacetic acid with overnight incubation at 4 °C. Further centrifugation at 11,000 rpm for 20 min resulted in a pellet containing extracellular proteins which was suspended in 50 μl of 1 M Tris–HCl buffer (pH 8), and dialyzed overnight against the same buffer. The freeze-dried protein content was determined as described by Lowry et al. (1951). The purified protein was ascertained as endotoxin free with the Limulus amebocyte lysate (LAL) test.

Animals

Male MF1-albino mice (5–6 weeks old; weighing 18–25 g; King Fahd Specialist Medical Centre, Jeddah, KSA) were used in the experiments and housed in a barrier room under standard conditions. The animals were kept in wire-mesh polycarbonate cages with autoclaved bedding, were acclimatized to laboratory conditions (12 h dark:12 h light cycles; 24.0 ± 1.0 °C) and had free access to food and water ad libitum. The food containers were refilled daily with fresh food and were fitted with bars to reduce losses. Routine clinical observations and body weight were measured regularly throughout the experiments. The mice were fed a standard diet for 2 weeks. Animal use and the care protocol were approved by the Research Ethics Committee, Taif University, Saudi Arabia.

Natural products

Hesperidin (HES) and ellagic acid (EA) used in this study were of analytical grade and purchased from Sigma Chemical Co. USA (Cat # H5452 and E2250, respectively).

Antibacterial activity

Agar–disk-diffusion

The disk diffusion method was employed for the determination of the antibacterial activity of hesperidin and ellagic acid against *A. hydrophila* according to Nostro et al. (2000). About 100 μl of *A. hydrophila* 1×10^6 CFU/ml was spread on solid medium plates. Filter-paper-disks were impregnated with 20, 40 or 60 μl of either HES (100, 50, 25 and 12.5 mg/ml) or EA (50, 20, 10, 5 and 2.5 mg/ml). The inoculated plates were incubated at 37 °C for 24 h. Each test was repeated four times, and the antibacterial activity was expressed as the mean of the inhibition zone diameters (mm) produced by the plant extracts.

Determination of minimal inhibition concentration (MIC)

The minimum inhibition concentration of the HES and EA was determined by the broth micro dilution method as described by Basri and Fan (2005) and Al-Bakri et al. (2009). HES and EA concentrations were two fold serially diluted in LB broth media in 96-wells of microtiter plates. Thereafter, 100 μl inoculum 1×10^6 CFU/ml of *A. hydrophila* in broth was added to each well. The microtiter plates were incubated at 30 °C for 24 h. Each extract was assayed in duplicate and each time two sets of microplates were prepared, one was kept for incubation while another set was kept at 4 °C to enable the turbidity in the wells of the microplates to be compared (the assessment of which was performed visually). The MIC values were taken as the lowest concentration of the extracts in the wells of those microtiter plates that showed no turbidity after incubation.

Determination of minimum bactericidal concentrations (MBC)

The MBC was determined according to the established methods of Shanholtzer et al. (1984), Fasching et al. (1990) and Irkin and Korukluoglu (2007). Tubes from the MIC experiment showing no turbidity were shacked and a 100 μl aliquot was transferred to an antibiotic-free nutrient agar plate and then spread over the plate by the lawning technique. The plates were incubated for 24 h at 30 °C. After the incubation period, the lowest concentrations of the extract that did not produce any bacterial growth on the solid medium were regarded as the MBC for this extract (Irkin and Korukluoglu, 2007). This observation was matched with the MIC test tube that did not show evidence of growth after 48 h of incubation.

Protective effect of hesperidin and ellagic acid against A. hydrophila using experimental mouse infection module (in vivo study)

The design of this experiment was intended to compare the efficacies of different herbal compounds for the treatment of mice infected with *A. hydrophila*. There were four experiment groups with ten mice in each group, including control [naive], bacteria-infected, hesperidin-treated (HES-B; 250 mg/kg/week HES) and ellagic acid-treated group (EA-B; 150 mg/kg/week EA). While, for survival studies, mice were