**Original article**

**In vitro and in vivo bioluminescent imaging to evaluate anti-Escherichia coli activity of Galla Chinensis**

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**A R T I C L E  I N F O**

Article history:
Received 6 March 2013
Received in revised form 12 March 2013
Accepted 11 April 2013
Available online 23 May 2013

Keywords:
bioluminescent imaging
*Escherichia coli*
Galla Chinensis

**A B S T R A C T**

**Background:** Novel and rapid technology is urgently needed to expedite screening of new antibacterial drugs for preventing and treating life-threatening bacterial infection.

**Purpose:** This study applied bioluminescent imaging to evaluate the antibacterial activity of *Galla Chinensis* extract (GCE) in gastrointestinal tracts of mice.

**Methods:** Bioluminescent *Escherichia coli* (*E. coli*) was constructed by transforming plasmid DNA containing TATA box-driven luciferase gene. The antibacterial activity of GCE in vitro was evaluated by mixing GCE and bioluminescent *E. coli* and subjecting to image; the antibacterial efficacy of GCE was evaluated *in vivo* by administering intragastric GCE and bioluminescent *E. coli* to mice and subjecting to image at 2 hours.

**Results:** GCE inhibited the growth of *E. coli* in a dose-dependent manner, with a minimal inhibitory concentration of 4 \( \mu \)g/mL and a minimal bactericidal concentration of 8 \( \mu \)g/mL. Intragastric administration of bioluminescent *E. coli* showed that a strong luminescent signal was observed in the abdominal region, while GCE treatment significantly decreased bioluminescence. *Ex vivo* image and bacterial count verified the decreased intensity of bioluminescence and number of viable *E. coli* in the intestines of GCE-treated mice.

**Conclusion:** The findings suggest: (1) GCE exhibits anti-*E. coli* activities in vitro and in vivo; and (2) the feasibility of bioluminescent imaging on real-time monitoring of the antibacterial effects of natural products in living mice.

**1. Introduction**

*Escherichia coli* (*E. coli*), a predominant species among facultative anaerobic bacteria in the gastrointestinal tract, is associated with a range of diseases: e.g., sepsis, urinary tract infection, meningitis, and gastroenteritis. Antibiotics typically control symptoms of *E. coli* infections. However, intensive use of antibiotics has led to emergence of resistant bacterial strains, meaning antibiotic therapy is not a viable solution for the treatment of *E. coli* infection, especially in endemic areas.
Plants are the most common sources of novel antibacterial remedies. Numerous studies deal with screening of antibacterial activity of medicinal herbs [5–8]. However, these studies report in vitro but not in vivo antibacterial efficacy via either the disc diffusion method or the tube dilution method. Moreover, bacterial viability and physical conditions, like osmotic pressure and pH, affect antibacterial efficacy; antibacterial activity of herbs in vitro does not guarantee that they work well in vivo [9]. The in vivo bioluminescent imaging system is a sensitive and noninvasive technique for rapid real-time detection of therapeutic efficacy. This technique has been used for monitoring bacterial infection [10–13] and assessment of host inflammatory responses to natural products [14–21]. Therefore, this study applied an in vivo imaging model for evaluating the antibacterial efficacies of medicinal herbs.

Galla Chinensis is a gall caused by a Chinese sumac aphid (Schlechtendalia chinensis Bell) on several Rhus species (Anacardiaceae), including Rhus chinensis Mill. Galla Chinensis has been used in traditional Chinese and other oriental medicine to treat diarrhea, prolonged coughing, and spontaneous perspiration [22]. We demonstrated earlier that Galla Chinensis extract (GCE) can suppress E. coli enterotoxin-induced diarrhea by blocking the binding of toxin to cellular receptors [23]. We wondered whether GCE could also suppress E. coli-induced diarrhea by killing bacteria and thus set up in vivo real-time imaging with bioluminescent E. coli, to evaluate GCE antibacterial ability. Findings suggest anti-E. coli activities are exhibited in vitro and in vivo. We also successfully set an in vivo real-time imaging to rate the antibacterial ability of GCE.

2. Materials and methods

2.1. Preparation of GCE

Galla Chinensis purchased from Sun Ten Pharmaceutical Corporation (Taipei, Taiwan) was ground to a fine powder and extracted by mixing 100 g of powder with 500 mL of water at 4 °C overnight. After centrifugation at 15,000 g for 15 minutes, the supernatant was collected, lyophilized, then stored at −30 °C for further analysis.

2.2. Bacterial strain, plasmid, and culture conditions

Our study used E. coli NM522 and pLuc-TATA as the bacterial strain and plasmid DNA, respectively. The latter was constructed by inserting firefly luciferase gene (Stratagene, La Jolla, CA, USA) into a plasmid containing TATA box (Fig. 1). For electroporation, a mixture containing 40 μL of cooled competent cell suspension and 1 μg of DNA was transferred into a precooled electroporation cuvette with a 0.1 cm electrode gap and electroporation was performed with Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA) at the following settings: peak voltage = 1.25 kV; capacitance = 25 μF; and parallel resistance = 600 Ω. After pulsing, the cells were immediately diluted with 1 mL of Luria-Bertani (LB) broth and incubated with agitation at 37 °C for 1 hour before plating onto LB agar containing 200 μg/mL ampicillin.

2.3. Susceptibility test

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of GCE were derived by the tube dilution method. A final inoculum of 106 colony forming units (CFU) per mL was used. MIC and MBC were defined as the lowest concentrations completely inhibiting E. coli growth and killing >99.9% of E. coli, respectively.

2.4. 3-(4,5-Dimethyl-2-thiazyl)-2,5-diphenyltetrazolium bromide (MTT) assay

The viability of E. coli was monitored by the MTT colorimetric assay [24]. Briefly, 100 μL of mixture containing 106 CFU/mL E. coli and various amounts of GCE was added to each well of 96-well culture plates and incubated for 24 hours at 37 °C. Ten microliters of 5 mg/mL MTT were added to the culture medium. After incubation at 37 °C for 4 hours, an equal cell culture volume of 0.04 N HCl in isopropanol was added to dissolve MTT formazan and absorbance at 570 nm was determined.

2.5. In vitro detection of bioluminescent signals

Bioluminescent bacteria cultured overnight at 37 °C were serially diluted with fresh LB broth in 96-well plates. Their bioluminescent signals were obtained by incubating with 200 μg of D-luciferin for 15 minutes and monitoring by IVIS Imaging System® 200 Series (Xenogen, Hopkinton, MA, USA) for 1 minute. Photons emitted from colonies of bioluminescent E. coli were detected by bioluminescent imaging system. Color overlay on image represents photons/second emitted from colony of bioluminescent E. coli, as indicated by color scale.
2.6. In vivo and ex vivo imaging of luciferase activity

Female BALB/c mice 8 weeks old, 20 ± 1 g weight were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Experiments were conducted under ethics approval from China Medical University Animal Care and Use Committee. For in vivo imaging, mice were intragastrically administrated with both \(10^7\) CFU of \(E.\) coli and 100 \(\mu\)g of D-luciferin, and/or various amounts of GCE. Two hours later, mice were anesthetized with isoflurane, placed face up in the chamber, and imaged for 1 minute with the camera set at the highest sensitivity by IVIS Imaging System\textsuperscript{R} 200 Series. Photons emitted from tissues were quantified by Living Image\textsuperscript{R} software. For ex vivo imaging, mice were sacrificed, tissues were rapidly removed and were imaged with the same settings used for in vivo studies. Signal intensity was quantified as the sum of detected photon counts per second from tissue (presented as photons/second).

2.7. Counting viable \(E.\) coli in intestines

Mice were intragastrically administered \(10^7\) CFU of \(E.\) coli and/or GCE. They were sacrificed 2 hours later, and their intestines were washed with 1 mL of saline. Serial dilutions of intestinal lavage fluid from each group were then inoculated onto LB agar plates containing 200 \(\mu\)g/mL ampicillin and incubated at 37°C for 18 hours for the bacterial count.

2.8. Statistical analysis

Intergroup differences were examined by the Student t test; a \(p\) value <0.05 was considered statistically significant.

3. Results

3.1. Construction and characterization of bioluminescent \(E.\) coli

We first constructed recombinant \(E.\) coli which constantly expressed luciferase genes. Fig. 1 shows 5.7-kb plasmid pLuc-

Fig. 2 — Comparison of bioluminescent imaging and MTT method for detecting viable \(E.\) coli. (A) Bioluminescent imaging. Various numbers of viable \(E.\) coli were imaged by IVIS system after adding D-luciferin. Color overlay on image represents photons/second emitted from \(E.\) coli, as indicated by color scale. (B) Quantification of photon counts by bioluminescent imaging (solid squares) and absorbance values at 570 nm by MTT assay (open squares), values are mean ± standard error of three independent assays.

Fig. 3 — Antibacterial effect of \(Galla\) Chinensis extract (GCE) in vitro. (A) MTT method. \(E.\) coli \((10^7\) CFU/well) was cultured in 96-well plates and treated with various amounts of GCE for 24 hours at 37°C. Data are expressed as absorbance values at 570 nm, values are mean ± standard error of triplicate assay. (B) Bioluminescent imaging. Various amounts of GCE were incubated with bioluminescent \(E.\) coli \((10^6\) CFU/well) for 4 hours at 37°C. After addition of D-luciferin, the 96-well plate was imaged. Color overlay represents photons/second emitted from the well, as indicated by color scale. Quantification of photon emission is shown at the bottom, values are mean ± standard error of three wells. \(*p < 0.05, **p < 0.01, ***p < 0.001,\) compared with mock.
TATA carrying TATA box (5’-TATATA-3’) and firefly luciferase gene. Recombinant plasmid was transferred to E. coli by electroporation. The bioluminescent signal of recombinant E. coli was monitored by the IVIS system after the addition of substrate D-luciferin. Fig. 2A plots the photon count increasing with the number of bioluminescent E. coli, suggesting that the intensity of the luminescent signal is directly proportional to the bacterial number. We further compared the sensitivity of the MTT colorimetric method with that of in vitro imaging. Fig. 2B shows limitations of the MTT method and bioluminescent image: 1.25 × 10^6 CFU/mL and 1.6 × 10^5 CFU/mL, respectively. Findings suggest that the sensitivity of bioluminescent imaging for the detection of bioluminescent E. coli increased 10-fold, as compared with the traditional MTT method.

3.2. In vitro antibacterial effect of GCE

The antibacterial effect of GCE was first analyzed by the tube dilution test. Data showed MIC and MBC of GCE against E. coli as 4 and 8 μg/mL, respectively. The antibacterial efficacy of GCE was further determined by the MTT method and bioluminescent imaging. E. coli was cultured in 96-well plates with various amounts of GCE, and the number of viable E. coli was monitored by MTT or luminescent imaging. Absorbance values at 570 nm (Fig. 3A) and luminescent intensities (Fig. 3B) dropped as GCE concentrations rose, suggesting that GCE inhibited the growth of E. coli in a dose-dependent manner. Additionally, GCE killed almost 100% of E. coli at 8 μg/mL, judged by MTT and bioluminescent imaging. The correlation between the tube dilution test and bioluminescent imaging suggests that luminescent imaging is a rapid method for evaluating the antibacterial effects of natural products.

3.3. Assessing luminescent signal after intragastrically administering bioluminescent E. coli in live mice

To monitor E. coli bioluminescent signals, live mice were inoculated intragastrically with 10^7 CFU of E. coli and 100 μL of 10^6 CFU/mL bacteria and 100 μL of 1 μg/μL D-luciferin, with signals monitored at indicated periods. Fig. 4 depicts strong luminescence in the

![Fig. 4 – Real-time monitoring of bioluminescent Escherichia coli dynamics in live mice. Mice were inoculated intragastrically with 10^7 CFU of E. coli and 100 μg of D-luciferin. Bioluminescent signal was monitored at intervals by IVIS imaging. Color overlay on the image represents photons/second emitted from the animal, as indicated by color scale. Photos are representative images of three independent experiments.](image-url)
mouth and abdominal regions at 10 minutes. Signals were mainly localized in the abdominal region at 1 hour, diffused to the anus at 3 hours and mainly localized in the anus region at 7 hours. The data portend in vivo bioluminescence to monitor the progression of bacterial infection in living mice.

3.4. In vivo antibacterial effect of GCE

To evaluate the in vivo effect of GCE on E. coli, we intra-gastrically administrated mice with 100 μL of 10⁶ CFU/mL E. coli, 100 μL of 1 μg/μL D-luciferase, and/or 100 μL of 8 μg/μL GCE, equivalent to MBC of GCE. The luminescent signal was monitored at 2 hours. Fig. 5A shows inoculation of bioluminescent E. coli displaying a strong luminescent signal in the abdominal region; GCE treatment decreased bioluminescence in the abdominal region. Ex vivo imaging further displayed the strongest bioluminescent signal in the small intestines of the E. coli group; bioluminescence in the small intestine declined in the GCE treatment group (Fig. 5B). Results indicate that GCE inhibited the growth of E. coli and killed E. coli in vivo. Fig. 5C records viable bioluminescent E. coli recovered from the intestinal tract. The totals of viable bacteria recovered from the intestines of the GCE, E. coli, and mock groups were 3.2 ± 0.21, 6.44 ± 0.05, and 0 log_{10} CFU, respectively. The number of viable bacteria in the intestinal tracts of GCE-treated mice was reduced 1000-fold compared with the E. coli group. These indicate that GCE exhibited antibacterial activity in vitro and in vivo; the correlation between bioluminescent imaging and the bacterial count suggests the feasibility of bioluminescence to rate the antibacterial activity of medicinal herbs.

4. Discussion

This study applied bioluminescent imaging to monitor the antibacterial effect of GCE. Traditional animal models of antimicrobial agents in vivo are inconvenient, require sacrifice at relevant time points, and assays may be influenced by tissue sampling techniques. Such studies are often expensive and labor-intensive, suggesting that novel in vitro and in vivo technology for rapidly screening novel antibacterial drugs is urgently needed to develop new drugs. Some studies emphasize the advantages of bioluminescent imaging in vivo for real-time monitoring of bacterial infection and drug therapy [10–13,25–27]. This is more sensitive and rapid than standard techniques, e.g., the disk diffusion test, and the tube dilution test. Moreover, bioluminescent imaging works well in real-time monitoring of bacterial infection in vivo. Kadurugamuwa et al used bioluminescent bacteria for monitoring biofilm infection in animals [25]. Xiong et al applied it to assess the efficacy of antibiotics in Staphylococcus aureus-induced endocarditis in rats [26]. Jawhara and Mordon employed bioluminescent E. coli to monitor bactericidal action in cutaneous wound infection [27]. Yet to date, bioluminescent imaging has never been utilized to monitor bacterial progression or drug therapy in intestinal E. coli infection. Our study used E. coli carrying pLuc-TATA plasmid to evaluate the in vivo antibacterial activity of GCE in intestines. Maximal bioluminescent signals could be detected 10 minutes after intragastric administration of E. coli; signals were mainly localized in the intestines at 2 hours. Hence, the in vivo antibacterial effect appeared at 2 hours in this model. GCE significantly suppressed the bioluminescent signal and number of viable E. coli in mice. The findings affirm the antibacterial effect of GCE in living mice by bioluminescent imaging.
The colorimetric method using MTT dye has served to test the susceptibility of antibacterial drugs [28]. This method displays excellent agreement with standard macrodilution [29], yet colors of herbal extracts may influence the accuracy of the MTT assay and limit its usage in herbal studies. At present, only a few studies find that herbs display fluorescence or interact with luciferin, a compound isolated from fireflies as substrate for luciferase. Besides the intrinsic low background of luminescence techniques, luciferase gene expression is just detected at very low levels in mammals [30]. The sensitivity of bioluminescent imaging showed a 10-fold increase over the traditional MTT method in detecting bacteria; intrinsic low background of luminescence techniques, luciferase display fluorescence or interact with luciferin, a compound isolated from fireflies as substrate for luciferase. Besides the intrinsic low background of luminescence techniques, luciferase gene expression is just detected at very low levels in mammals [30]. The sensitivity of bioluminescent imaging showed a 10-fold increase over the traditional MTT method in detecting bacteria; in vitro antimicrobial susceptible data were rapidly obtained by bioluminescent imaging, which in this study affords a rapid and reliable means to evaluate the antibacterial activities of herbs. Galla Chinensis has seen extensive use in traditional Chinese medicine to treat diarrhea, prolonged coughing, and spontaneous perspiration [22]. It shows promise in preventing dental caries by inhibiting the cariogenicity of oral biofilm (Streptococcus sanguis, Streptococcus mutans, Actinomyces nae-slundi, and Lactobacillus rhamnosus) [31,32]. Galla Chinensis reportedly inhibits in vitro growth of bacteria [33–36]. Our prior study showed how gallic acid from Galla Chinensis blocks the binding of bacterial toxin to cellular receptors and suppresses toxin-induced diarrhea [23]. This study applied bioluminescent imaging to rate the therapeutic efficacy of GCE in E. coli-infected mice; the data indicate that GCE suppresses the growth of E. coli in vivo. The findings suggest Galla Chinensis for treating diarrhea, by both blocking bacterial toxin and killing bacteria. In summary, Galla Chinensis has long been used for the treatment of diarrhea. Our earlier study demonstrated that GCE suppresses E. coli toxin-induced diarrhea by blocking the binding of toxin to cellular receptors [23]. This study set up in vivo real-time imaging with bioluminescent E. coli and found that GCE suppressed the growth of E. coli and killed E. coli in vitro and in vivo. The findings also suggested in vivo bioluminescent imaging as a rapid and reliable approach for the assessment of antibacterial agents from medicinal herbs.

Acknowledgments

This work was funded by grants (NSC101-2325-B-039-007) from the National Research Program for Biopharmaceuticals, National Science Council, and (CMU101-S-21 and CMU101-AWARD-09) China Medical University.

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