In vitro synergistic potentials of novel antibacterial combination therapies against Salmonella Typhimurium and Escherichia coli

Md. Akil Hossain(Former Corresponding Author)
  Animal and Plant Quarantine Agency

Hae-Chul Park
  Animal and Plant Quarantine Agency

Kwang-jick Lee
  Animal and Plant Quarantine Agency

Sung-Won Park
  Animal and Plant Quarantine Agency

Seung-Chun Park
  Kyungpook National University

JeongWoo Kang(New Corresponding Author)

Research article

Keywords: Combination therapy, critically important antibiotics, Phenolic compounds, gallic acid, hamamelitannin, biofilm.

Version of Record: A version of this preprint was published at BMC Microbiology on May 14th, 2020. See the published version at https://doi.org/10.1186/s12866-020-01810-x.

Abstract

Background: Bacteria have remarkable abilities to acquire resistance against antibiotics by several mechanisms. New strategies are needed to block the development of resistance and to prolong the life of traditional antibiotics. This study aimed to increase the efficacy of existing antibiotics by combining them with the opportunistic phenolic compound gallic acid (GA) and its derivatives. Fractional inhibitory concentration (FIC) indexes of phenolic compound-antibiotic combinations against Salmonella enterica serovar Typhimurium (S. Typhimurium) and Escherichia coli (E. coli) were determined. Based on the FIC index values and clinical importance, 3 combinations were selected to evaluate their effects on the virulence factors of these bacteria. The in vitro cytotoxicity of GA and hamamelitannin in human (Hep G2) and Rattus norvegicus (IEC-6) cell lines were evaluated. Results: Phenolic compounds demonstrated considerable antibacterial effects as the minimum inhibitory concentrations (MICs) of epigallocatechin, GA and hamamelitannin found against different strains were (512−1024), (256−1024) and (512−2048) µg/mL, respectively. The FIC indexes of the combined antibacterials against these strains were 0.281− 1.016. The time-kill assays and ultrastructural morphology showed that the GA-ceftiofur combination, and hamamelitannin-erythromycin and GA-ampicillin combinations more efficiently inhibited the growth of S. Typhimurium and E. coli , respectively, compared to the individual antibiotics. Biofilm viability and the swimming and swarming motilities of S. Typhimurium in the presence of GA-ceftiofur and E. coli in the presence of the hamamelitannin-erythromycin and GA-ampicillin combinations were more competently inhibited than individual antimicrobials. The inhibitory concentrations 50% (IC 50) of GA and hamamelitannin in Hep G2 cells were 1427.34 µM and 2024.97 µM, and in IEC-6 cells were 564.55 µM and 988.54 µM, respectively. Conclusions: We can conclude that these antibacterial combinations can be potential medications to treat S. Typhimurium and E. coli infections. Further study to confirm this effect in in vivo system and to determine the precise mechanism of action should be undertaken to establish these combinations as medications.

Background

Infectious diseases are the third most significant cause of mortality around the world according to the World Health Organization (WHO) [1]. Multidrug-resistant (MDR) bacteria are one of several vital aetiologic agents contributing to the emergence of infections [2]. The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics [3−8]. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements [4−7, 9−16]. The Centers for Disease Control and Prevention (CDC) has classified a number of bacteria as presenting urgent, serious, and concerning threats, many of which are already responsible for placing substantial clinical and financial burden on the health care system, patients, and their families [3, 7, 12, 17].

The frequency of resistance is observed equally among Gram-negative and Gram-positive organisms, although Gram-negative bacteria are more prone to develop the MDR phenotype [2]. *Together with other bacterial species, Escherichia coli* and *Salmonella Typhimurium* are severely antibiotic-resistant and were recently enlisted and designated as priority class bacterial pathogens in urgent need of effective antibiotics

by the WHO [18]. The gravity of the situation is highlighted by the fact that clinical isolates of these species have up to 1000-fold higher 50% growth inhibition concentrations (GIC_{50}) for a range of antibiotics with different mechanisms of action relative to the sensitive/resistant breakpoints recommended by the Clinical and Laboratory Standard Institute (CLSI). These trends show the urgent need for the development of new antimicrobials that can treat or potentiate current antibiotics against MDR bacteria [19]. The scientific community is continuously searching for new classes of disinfection systems that could act efficiently against these pathogens [20]. Certain naturally occurring phenolic compounds have antioxidant, anticarcinogenic, and antimicrobial activities [21, 22].

The phenolic compound (methyl gallate and pyrogallol)-containing *Nymphaea tetragona* 50% methanol extract (NTME) was found to have quorum sensing and virulence factor inhibitory effects [23]. The synergistic antibacterial and quorum sensing (QS) inhibition effects of the phenolic compound-containing NTME were also evident in our earlier study [24]. The phenolic compound gallic acid demonstrated the potential to inhibit *S. mutans* biofilms [25]. Recently, we also reported that methyl gallate, a gallic acid derivative, can efficiently interfere with the QS regulatory pathways of *P. aeruginosa* and inhibit the adhesion, invasion and intracellular survival of *S.* Typhimurium [26, 27]. These properties of bacteria are known to have a significant role in increasing pathogenicity and antimicrobial resistance [28].

Gallic acid derivatives contain a large number of hydroxyls, which can form protonic and ionic bonds and combine with many biological proteins, such as enzymes, carriers, ion channels and receptors, deactivating them and consequently exhibiting bacterial inhibition. Additionally, many phenols can non-specifically affect molecular targets of microorganism [29]. These observations initiate the speculation that gallic acid derivatives may also potentiate the efficacy of existing antibiotics. Thus, we intended in this study to evaluate the antibacterial potentials of gallic acid and its 4 derivatives individually; and in combination with 8 commercially available antibiotics against *S.* Typhimurium and *E. coli*. Additionally, the effects of those combination antibacterials against selected virulence factors, including biofilm formation and motility were determined. Finally, the safety profiles of potent phenolic compounds were investigated for further commercial use.

Materials And Methods

Chemicals, reagents and bacterial strains

Luria-bertani (LB), mueller hinton agar (MHA), mueller hinton broth (MHB); cation-adjusted (CA)-MHB, nutrient broth (NB), trypticase soy broth (TSB), and agar were purchased from Becton Dickinson and Company (Becton Drive, NJ, United States), and sodium chloride and glucose were obtained from Scharlab (Barcelona, Spain). Quality control strains of *E. coli* (ATCC 25922) and *S.* Typhimurium (ATCC 14028), and clinical strains of *S.* Typhimurium (V08-S-HA-06-170, V15-S-HA-02-210, SAL 109, SAL 202 and SAL 224) were used in this study. Clinical strains used in this study were obtained from farms of different regions in the Republic of Korea. All the strains were cultivated in their respective growth media for 20 h in a rotating incubator at 200 rpm and 37 °C. Antibiotics used in this study include amoxicillin, ampicillin, cefotaxime, ceftiofur, erythromycin, florfenicol, marbofloxacin, norfloxacin, penicillin G and thiamphenicol. Galic acid,

epicatechin, epicatechin gallate, epigallocatechin and hamamelitannin were utilized as antibacterial agent in this study. All the chemicals, reagents and media were from Sigma-Aldrich (St. Louis, MO, United States) unless otherwise mentioned.

Minimum inhibition concentrations of antibacterial agents

MICs of above mentioned commercial antibiotics and opportunistic antibacterial agents were determined by the standard broth microdilution method according to the CLSI guidelines [30] in CA-MHB using an inoculum concentration of ∼5×10^5 CFU/mL. Different antibacterial solutions were serially diluted in 96-well plates in 100 µL volumes. The cultures of different bacterial strains were diluted to adjust 0.5 McFarland units and, again diluted 100-times. Hundred microliters of these diluted bacterial suspensions were dispensed to all the wells of 96-well plates which contain 100 µL of antibacterial solution. After incubation at 35 °C overnight, the turbidity in each well was checked. The lowest concentrations of the antibacterial that completely inhibited any increase in turbidity were considered as the MICs.

Fractional inhibition concentration index of antibacterial agents

A slightly modified version of the previously described checkerboard microdilution method was utilized to determine the combination interactions of the commercial antibiotics and phenolic compounds [31]. One antibacterial agent was vertically diluted and the other antibacterial was horizontally diluted in 96-well plates to achieve a matrix of different combinations of the 2 antibacterials. Similar dilutions of individual drugs and the drug-free medium control were included in each test plate. Bacterial cultures in early log phase were diluted and 100 µL of the diluted bacterial suspension was added to each well of the 96-well plates, where the final inoculum concentration after transferring to each well would be ∼5×10^5 CFU/mL. Plated bacteria were incubated at 35 °C for 16 to 20 h. The fractional inhibitory concentration (FIC) and the FIC index (FICI) were calculated from the MICs of the drugs alone and in combination. The FIC is the MIC of a drug in presence of another drug divided by the MIC of the individual drug, and the FICI is the sum of the FICs of the individual drugs. An FICI of ≤ 0.5 is regarded as synergistic, 0.5 < FICI ≤ 1 is considered additive, 1 < FICI ≤ 2 is considered indifferent, and an FICI > 2 is considered antagonistic effects [32].

Effect of antibacterial combinations on bacterial inhibition rates

The time-dependent inhibition effects of gallic acid-ceftiofur against *S.* Typhimurium and hamamelitannin-erythromycin and gallic acid-ampicillin against *E. coli* were evaluated according to a previously reported method [24]. Drug compounds alone and in combination were supplemented in 10 mL MHB broth in 15 mL falcon tubes. Bacterial cultures in early log phase were diluted and then resuspended in the drug-supplemented broth to a final inoculum concentration of 5×10^6 CFU/mL. A tube containing 5×10^6 CFU/mL of bacteria in 10 mL MHB without any drug was used as a control. The samples were incubated at 37 °C at 200 rpm in a shaking incubator. At different time points (0, 1, 2, 3, 4, 6, 8, 12, and 24 h) 100 µL of the cultures were collected from all tubes and serially diluted 10-fold in agar saline. Aliquots of the 10-fold dilutions (20 µL) were spread on Mueller Hinton agar (MHA) plates and incubated overnight at 37 °C. The

CFUs of the cultures were determined by counting the number of colonies from each dilution. The mean $\log^{10}$ CFU/mL for each compound was plotted against different times.

Effect of antibacterial combinations on bacterial cell morphology

The effects of the gallic acid-ceftiofur combination on the morphology of *S.* Typhimurium and the hamamelitannin-erythromycin and gallic acid-ampicillin combinations on the morphology of *E. coli* cells were evaluated. Drug compounds alone or in combination were supplemented into 10 mL of MHB broth in 15 mL falcon tubes. Bacterial cultures in early log phase were diluted and then resuspended in the drug-supplemented broth to a final inoculum concentration of 5×10^6 CFU/mL. A tube containing 5×10^6 CFU/mL of bacteria in 10 mL MHB without any drug was used as a control. The bacteria in tubes were incubated overnight at 37 °C and 200 rpm in a shaking incubator. Then, the cells were harvested, washed, and dehydrated according to a previously reported protocol [33]. The ultrastructural morphology of treated *S.* Typhimurium and *E. coli* cells was studied using a scanning electronic microscope (SEM; models S-4300 and EDX-350; Hitachi, Japan).

Effect of antibacterial combinations on biofilm growth and viability

The inhibitory effect of combination antibacterials on biofilm formation was determined using slightly modified version of a previously reported spectrophotometric method [34, 35]. Briefly, test compounds were supplemented into TSB in three separate wells of a 96-well microplate for each concentration. The final concentrations of the test compounds alone or in combination (gallic acid-ceftiofur, gallic acid-ampicillin and hamamelitannin-erythromycin) were ¼MIC, ½MIC and 1MIC after bacterial inoculation. Cultures of *S.* Typhimurium and *E. coli* were incubated for 18 h in a rotating incubator at 200 rpm and 37 °C. The bacterial cultures were diluted in TSB, and then 100 μL of the diluted cultures were added to the designated wells to a final cell density of 1×10^6 CFU/mL after inoculation. The optical densities of the bacteria in the wells of a 96-well plate were measured at 600 nm instantly after inoculating bacteria. The bacteria in the 96-well plate with drugs were incubated for 24 h at 37 °C, and after incubation, the optical densities were again measured to determine the growth of planktonic cells. Then the supernatants from the wells of a 96-well plate were discarded carefully without affecting the biofilms which are attached on the well-surfaces. The adherent media and drug components were removed by washing the wells three-times with sterile phosphate buffer saline (PBS, pH 7.2). Then, 200 μL of methanol (99%, v/v) were dispensed to the wells, and kept for 20 min to fix the biofilms. The biofilms were then stained by introducing 100 μL of crystal violet (0.2%, w/v) solution to the wells and keeping at room temperature for 15 min. The excess or unbound crystal violet in the wells was removed by four-times washing with PBS. The crystal violet on the biofilm cells was extracted in 100 μL of 95% ethanol, and their optical densities (OD) were measured, which yields a measure of biofilm formation (compared to the control). Measurements were performed in triplicate and repeated 3 times.

Previously, reported biofilm viability assay methods were utilized to evaluate the effects of combination drugs on the viability of the biofilms produced by *S.* Typhimurium and *E. coli* [23, 36]. In brief, sterile TSB broth of 2 mL were transferred to a Nunc™ Lab-Tek™ II Chambered Cover glass (ThermoFisher Scientific, Waltham, MA, United States), and diluted cultures of *S.* Typhimurium and *E. coli* were inoculated into the

broth to a final concentration of 1×10^6 CFU/mL. The Nunc™ Lab-Tek™ II Chambered Cover glass which contain *S.* Typhimurium and *E. coli* cells were kept in a static incubator at 37 °C until 48 h for biofilm formation. Every 24 h, the TSB broth used in biofilm formation was replaced by fresh, sterile TSB broth. The supernatants and planktonic cells were discarded after incubating the bacteria for 48 h, and the chambered cover glasses were washed by 1× PBS. Then, 2 mL of sterile TSB containing the test compounds alone or in combination (gallic acid-ceftiofur, gallic acid-ampicillin and hamamelitannin-erythromycin) at ¼MIC, ½MIC and 1MIC concentrations were added. The biofilms were again kept in a static incubator at 37 °C for 24 h to treat the developed-biofilm cells. After 24 h of exposure to the test compounds, the biofilms were again washed with sterile double distilled water (DDW) and stained with BacLight live/dead stain (ThermoFisher Scientific, Waltham, MA, United States). Confocal laser scanning microscope (CLSM) was used to scan the viable and nonviable biofilms. Imaging was performed with a Zeiss LSM 700 CLSM (Zeiss, Oberkochen, Germany) within the 488 nm excitation and 560−600 nm emission range. Zen 2009 software was used to execute image acquisition as well as subsequent image manipulation. Untreated biofilm was used as a control.

Effect of antibacterial combinations on the motility of bacterial cells

The swarming and swimming motilities of *E. coli* (ATCC 25922) and *S.* Typhimurium (ATCC 14028) in the presence of the combination drugs were evaluated according to previously published methods with slight modifications [37, 38]. The media used for the *E. coli* (ATCC 25922) swarming assay was composed of 0.8% Luria-Bertani (LB) supplemented with 0.5% glucose and 0.6% agar. Nutrient broth (NB) supplemented with 0.5% glucose and 0.5% agar was used for the evaluation of *S.* Typhimurium (ATCC 14028) swarming motility. The media used to evaluate *E. coli* swimming activity was composed of 1% tryptone broth supplemented with 0.5% NaCl and 0.3% agar. Nutrient broth supplemented with 0.5% glucose and 0.25% agar was used as the media for the *S.* Typhimurium swimming motility assay. Molten agar plates were supplemented with ¼MIC, ½MIC and 1MIC concentrations of test compounds alone or in combination (gallic acid-ceftiofur, gallic acid-ampicillin and hamamelitannin-erythromycin). A non-supplemented drug free plate was employed as the negative control. The plates were allowed to dry for 1 h and then 2 µL of *E. coli* and *S.* Typhimurium cultures were inoculated onto the respective swarming and swimming agar plates. For both strains, swarm plates were kept at 37 °C overnight, whereas swim plates were incubated at 37 °C for 10 h. After incubation, the swarm and swim zone diameters were measured using calibrated digital slide callipers (Mitotoyo, Japan), and photographs of the plates were captured.

Cell viability in the presence of antibacterial agents

The *in vitro* viability of human hepatocyte (Hep G2; American Type Culture Collection HB-8065, VA, United States) and *Rattus norvegicus* small intestine (IEC-6; American Type Culture Collection CRL-1592, VA, United States) cell lines in the presence of gallic acid, hamamelitannin, ampicillin, ceftiofur and erythromycin alone, and combinations of gallic acid-ceftiofur, gallic acid-ampicillin and hamamelitannin-erythromycin were evaluated according to standard EZ-cytox (EZ-1000; Daeillab Service Co. Ltd., Jeonju, South Korea) assay method. In brief, Hep G2 cells were cultured in foetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%) solution-supplemented Eagle's Minimum Essential Medium (EMEM;

ThermoFisher Scientific, Waltham, MA, United States) at 37 °C under a humidified atmosphere of 5% carbon dioxide (CO_2). Whereas, the IEC-6 cells were cultured at 37 °C under a humidified atmosphere of 5% carbon dioxide (CO_2) in Dulbecco's Modified Eagle's medium (DMEM; ThermoFisher Scientific, Waltham, MA, United States) with 4 mM L-glutamine (ThermoFisher Scientific, Waltham, MA, United States), adjusted to contain 1.5 g/L sodium bicarbonate (Carolina Biological Supply Company, Burlington, NC, United States) and 4.5 g/L glucose and supplemented with 0.1 Unit/mL bovine insulin (90%) and FBS (10%). Both types of cells were subpassaged at a ratio of 1:5 twice a week. One hundred microliters of suspended cells (2×10^4 cells/mL in their respective culture medium) were acclimated in 96-well plates at 37 °C under 5% CO_2 for 24 h. The medium from each well was aspirated and the cells were washed twice. One hundred microliters of the test compounds at various concentrations in their respective culture medium were dispensed into each well and the cells in the drug-supplemented medium were allowed to incubate at 37 °C under 5% CO_2 for 24 h. A total of 10 μL of EZ-cytox were added to each well. After incubation for 2 h, the absorbances in each well were measured at 450 nm using a plate reader. Cells not treated with any drugs were assigned as the control. The cell viability (%) was calculated by the following formula, and values of cell viability (%) at different drug concentrations were used to determine the inhibitory concentration 50% (IC_{50}):

Cell viability (%) = (OD of drug-treated sample/OD of untreated sample) × 100, where OD is the optical density [39].

Statistical analysis

Results are presented as the means ± standard deviation (SD) of triplicate analysis. Statistical analysis was carried out by using SAS software (SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) followed by F-test was used to compare the results. Statistical significance was considered when the *P*-value was <0.05.

Results

Antibacterial activities of commercial antibiotics and phenolic compounds

Antibacterial activities of different antibiotics and phenolic compounds were evaluated against quality control (QC) strains of *E. coli* and *S.* Typhimurium, and clinical strains of *S.* Typhimurium. The MICs of the different antibiotics (amoxicillin, ampicillin, cefotaxime, ceftiofur, erythromycin, florfenicol, marbofloxacin, norfloxacin, penicillin G and Theamphenicol) against QC strains of *E. coli* and *S.* Typhimurium ranged from 0.125~256.00 μg/mL and 0.062~128.00 μg/mL, respectively. In contrast, the MICs of the commercial antibiotics against the clinical isolates of *S.* Typhimurium ranged from 0.25 to ≥1024 μg/mL. The results in Table 1 clearly demonstrate that the MICs of almost all of these commercial antibiotics against the clinical isolates were increased by several folds, which indicates that resistance has developed in these clinical strains [40–44]. The MICs of phenolic compounds (epicatechin, epicatechin gallate, epigallocatechin, gallic acid and hamamelitannin) against the QC strains and clinical isolates of *S.* Typhimurium ranged from 256 to ≥1024 μg/mL, with gallic acid being the most potent among all the compounds. The MICs of the phenolic compounds against the QC strains of *E. coli* ranged from 512~2048.00 μg/mL.

In vitro synergy with commercial antibacterials

Checkerboard microdilution assays were performed to evaluate the combination antibacterial interactions of the commercial antibiotics with the phenolic compounds. The results of the combined activities are presented in Table 2. Synergistic effects against *E. coli* were obtained from the combinations of thiamphenicol and gallic acid (FICI: 0.281), erythromycin and hamamelitannin (FICI: 0.375), and thiamphenicol and hamamelitannin (FICI: 0.50). The combination of erythromycin and epicatechin gallate against *S.* Typhimurium also showed synergistic antibacterial effects (FICI: 0.50). Moreover, additive effects were obtained from many combinations against *S.* Typhimurium (FICI: 0.502~0.750; n=16) and *E. coli* (FICI: 0.502~0.625; n=17). The rest of the combinations showed indifferent effects against both bacteria, with no antagonistic effects observed from any of the combinations. Depending on both the clinical and commercial importance, 3 combinations that had synergistic or additive antibacterial effects were selected for further study.

Effects of combination drugs on time- and concentration-dependent inhibition

The inhibitory effects of the combination antibacterials on bacterial growth rates over time are presented in Figure 1. The growth rate of *S.* Typhimurium (ATCC 14028) was approximately the same when a 1MIC concentration of gallic acid was supplemented into the culture, and the cell density increased by approximately 1-fold at 24 h compared to the density at the time of inoculation (Figure 1a). The bacterium in the presence of 1MIC ceftiofur showed complete inhibition within 8 h, after which log phase was initiated. Finally, after 24 h, the cell density reached a level 1-fold higher than its initial density. In contrast, the cell number in the drug-free control culture increased by approximately 5-fold within 6 h, and the same cell density was sustained out to 24 h of incubation. Treatment with the antibacterial combination (1MIC of both ceftiofur and gallic acid) completely inhibited the bacterial growth by 12 h, after which time the bacteria gradually revived and finally reached a cell density at 24 h that was 2-fold less than its initial 6-fold density. Sub-MIC levels of both drugs in combination prevented this increase in growth such that the final cell densities at 24 h were almost the same as their initial cell densities.

At 24 h, the density of hamamelitannin (1MIC)-treated *E. coli* (ATCC 25922) cells was approximately 6-fold less than the drug-free control. The growth of the *E. coli* cells was also inhibited more than 6-fold at 24 h in the presence of erythromycin (1MIC) compared to the control. Compared to the control, approximately 5-fold less growth was observed after treating this bacterium for 24 h with sub-MIC levels of antibacterial combinations (½MIC of both hamamelitannin and erythromycin). Moreover, the ¼MIC of both drugs together inhibited the growth by approximately 4-fold, which demonstrated the potential of this combination drug for bacterial inhibition.

E. coli (ATCC 25922) cultures incubated with a 1MIC concentration of gallic acid for 24 h showed approximately 5-fold less growth than the drug-free control. Compared to the control, the growth of the *E. coli* cells was also inhibited by approximately 6-fold at 24 h in the presence of ampicillin (1MIC). Approximately 5-fold less growth was observed compared to the control after culturing this bacterium for 24 h with sub-MIC levels of the antibacterial combination (½MIC of both gallic acid and ampicillin).

Effects of combination drugs on the morphology of bacterial cells

The ultrastructural morphologies of *S.* Typhimurium (ATCC 14028) treated with the drug combination of gallic acid and ceftiofur and *Escherichia coli* (ATCC 25922) treated with 2 drug combinations (hamamelitannin-erythromycin and gallic acid-ampicillin) were studied to assess whether the combination drugs had any impact on the cellular architecture. The representative SEM images of gallic acid and ceftiofur-treated *S.* Typhimurium cells are shown in Figure 2. The SEM images revealed that untreated and gallic acid (1MIC)-treated *S.* Typhimurium cells had rod-like shape and were separated with perfect symmetry. In addition, binary fission of the bacteria was evident in the SEM images (Figure 2a and c). The cells treated with ceftiofur alone or in combination with gallic acid were found in a long rope-like shape, and no binary fission was evident, which is completely different from control cells. None of the cells were pitted, deformed or broken and the antibacterials had no effect on the cell wall or cytoplasmic membrane of the bacteria. The *E. coli* cells treated with hamamelitannin-erythromycin and gallic acid-ampicillin combinations also showed similar changes in cell length and binary fission without any effects on the cell wall or cytoplasmic membrane (data not shown).

Effects of combination drugs on biofilm inhibition and viability

S. Typhimurium (ATCC 14028) biofilm formation in the presence of the gallic acid-ceftiofur drug combination and *E. coli* (ATCC 25922) biofilm formation in the presence of the hamamelitannin-erythromycin and gallic acid-ampicillin drug combinations were evaluated using a static biofilm assay. The effects of the combination antibacterials on the growth of planktonic and biofilm cells of *S.* Typhimurium and *E. coli* are shown in Figure 3. The inhibition of both planktonic and biofilm cells of these bacteria was more induced by the combination drugs than by the individual drugs in most cases. The ¼MIC of the commercial antibiotics could significantly inhibit *S.* Typhimurium and *E. coli* biofilm formation when a lower amount of the respective phenolic compounds were applied together with these antibiotics.

The viability of the biofilms in the presence of the combination antibacterials was determined by staining the biofilms with BacLight live/dead stain and imaging with a CLSM. The reduction in biofilm viability with the combination antibacterial treatments is demonstrated by the results obtained with the CLSM. Figure 4 shows the CLSM images of *S.* Typhimurium and *E. coli* biofilms formed on a glass surface and treated with or without the combination antibacterials. The confocal micrograph of 48 h *S.* Typhimurium and *E. coli* biofilms treated with combination antibacterials for 24 h displays a lower proportion of live cells (cells that are stained green) than those observed in the untreated controls. It is clearly visible that almost all the cells were alive in the control biofilm, while most of the cells were dead (cells that are stained red) after treatment with the combination antibacterials.

Effects of combination drugs on the motility of bacterial cells

The effects of the antibacterial combinations on the swimming and swarming motilities of *S.* Typhimurium (ATCC 14028) and *E. coli* (ATCC 25922) were evaluated. Representative photographs of drug-treated swim and swarm plates are displayed in Figure 5. Table 3 shows the diameters of the swim and swarm zones. The results demonstrated that the hamamelitannin-erythromycin combination and the gallic acid-ampicillin

combination significantly inhibited both the swimming and the swarming motilities of *E. coli*. Similarly, the swimming and swarming motilities of *S.* Typhimurium were noticeably inhibited by the gallic acid-ceftiofur combination. Moreover, the combination antibacterials showed better inhibitions of swimming and swarming motilities at sub-MIC concentrations compared to their individual effects at MIC concentrations.

Safety profiles of potent phenolic compounds

The effects of gallic acid and hamamelitannin alone, and gallic acid-ampicillin, gallic acid-ceftiofur and hamamelitannin-erythromycin combinations on the viability of Hep G2 and IEC-6 cells are shown in Table 4 and Additional file 1. The IC_{50} of gallic acid and hamamelitannin in Hep G2 cells were 1427.34 µM and 2024.97 µM, and in IEC-6 cells were 564.55 µM and 988.54 µM, respectively. The IC_{50} values of phenolic compounds are more in presence of commercial antibiotics than IC_{50} values of those phenolic compounds alone (in absence of commercial antibiotics) in both types of cell lines. In contrast, IC_{50} values of commercial antibiotics are less in presence of phenolic compounds than IC_{50} values of those commercial antibiotics alone (in absence of phenolic compounds) in both cell lines. However, the hamamelitannin-erythromycin combination on IEC-6 cells showed opposite trend.

Discussion

There are recent reports indicating the resistance of several bacterial strains to different antibiotics that have been used in the treatment of infectious diseases of human [45]. Thus, to combat infectious diseases associated with resistant pathogens, the development of alternative antimicrobial drugs is urgently needed [46, 47]. The *in vitro* activities of all the tested phenolic compounds against resistant strains of *Salmonella* Typhimurium (Table 1) reflects that those compounds could be good candidates to minimize the development of bacterial resistance and to ensure clinical treatment of bacterial infections. In the present study, the MIC results demonstrated the antibacterial activities of the phenolic compounds against all the tested strains of *S.* Typhimurium and *E. coli,* which have been shown to be resistant to one to eight out of ten currently available antibiotics (Table 1). The potentials of these phenolic compounds were further explored through their combined interactions with commercial antibiotics, where they possessed synergistic effects with thiamphenicol and erythromycin against *S.* Typhimurium and *E. coli* and additive and/or indifferent effects with the other tested antibiotics. The time- and concentration-dependent inhibition assays also exposed that the combinations of phenolic compounds and commercial antibiotics more effectively inhibited the growth of *S.* Typhimurium and *E. coli* than the antibiotics alone. Furthermore, the combinations of phenolic compounds and commercial antibiotics demonstrated improved inhibition of biofilm formation and motility in *S.* Typhimurium and *E. coli*.

The MICs of the 5 phenolic compounds (epicatechin, epicatechin gallate, epigallocatechin, gallic acid and hamamelitannin) were investigated against QC strains of *S.* Typhimurium and *E. coli*, and 5 clinical strains of *S.* Typhimurium. It has been reported that gallic acid can restrain the growth of many bacteria, including methicillin-sensitive *S. aureus*, MRSA, *E. coli, P. aeruginosa*, and *Salmonella typhi* [48]. The results in Table 1 indicate that gallic acid possessed the strongest antibacterial activity among these phenolic compounds, followed by epigallocatechin, hamamelitannin, epicatechin gallate and epicatechin. Moreover, very

convincing MIC values were obtained for gallic acid and epigallocatechin against *S.* Typhimurium and *E. coli*, and even for hamamelitannin against *S.* Typhimurium. The MIC values of gallic acid against *S.* Typhimurium (256 μg/mL) and *E. coli* (1024 μg/mL) in this study are lower than those reported previously (2500 μg/mL) [48]. The mean MICs of plant-derived epigallocatechin against *S.* Typhimurium and *E. coli* were reported to be 572±186 and 733±121 μg/mL, respectively [49]. The MIC values of pure epigallocatechin against *S.* Typhimurium (512 μg/mL) and *E. coli* (512 μg/mL) in our study were lower than the previously reported MIC values [49]. This lower MIC value for plant-derived epigallocatechin against *S.* Typhimurium and *E. coli* in the previous study compared to the MIC value for pure epigallocatechin in our study is might be because of the purity of the compound used. However, the MIC values for plant-derived epigallocatechin against *S.* Typhimurium and *E. coli* were comparable with the results of our study. Likewise, the MICs of epicatechin against *S.* Typhimurium and *E. coli* were 2500 μg/mL, which demonstrates the similarity between our results and previously published results [48].

It is always recommended to treat bacterial infections with a combination of antimicrobial agents to prevent the development of drug resistance and to improve efficacy. Drug combinations with synergistic interactions are generally considered to be more effective and, therefore, preferable [50]. Incidentally, synergistic effects were obtained from the combinations of thiamphenicol and gallic acid (FICI: 0.281), erythromycin and hamamelitannin (FICI: 0.375), and thiamphenicol and hamamelitannin (FICI: 0.50) against *E. coli*. The combination of erythromycin and epicatechin gallate against *S.* Typhimurium also showed synergistic antibacterial effects (FICI: 0.50). Moreover, additive effects were obtained from many combinations against *S.* Typhimurium (FICI: 0.502~0.750; n=16), and *E. coli* (FICI: 0.502~0.625; n=17). The rest of the combinations had indifferent effects against these bacteria. Excellent *in vitro* activity combined with the synergistic effects with other antibacterial drugs underscores the potential utility of these phenolic compounds for the treatment of *E. coli* and *S.* Typhimurium-associated infections. The combinations of gallic acid and ceftiofur, hamamelitannin and erythromycin, and gallic acid and ampicillin, which have synergistic or additive antibacterial effects, against *S.* Typhimurium and *E. coli* were selected for further studies depending on both the clinical and commercial importance of each combination.

Time-kill assays are useful for the evaluation of the pharmacodynamic characteristics of new antimicrobial agents and to determine whether the effects of antibacterials are bacteriostatic or bactericidal [51]. According to our results, gallic acid and hamamelitannin alone or in combination with ceftiofur, ampicillin and erythromycin show bacteriostatic activity against the tested bacteria, as a reduction ≥99.9% of the inoculum was not observed compared to the growth control. At ½MIC, both gallic acid and ceftiofur in combination were able to inhibit *S. typhimurium* in the first 8 h of incubation, similarly the 1MIC concentration of both drugs in combination demonstrated complete inhibition within the first 8 h. The bacteria treated with ½MIC and 1MIC concentrations of the combination antibacterials started their log phase after the first 8h and 12 h of incubation, respectively. At the end of the incubation period (24 h), a greater than 3-fold reduction in the inoculum concentration with ½MIC of gallic acid and ceftiofur and an approximately 7-fold lower inoculums concentration with 1MIC of gallic acid and ceftiofur were achieved in contrast to the control. The 1MIC of both hamamelitannin and erythromycin together inhibited the growth of *E. coli* completely within the first 12 h, after which the bacteria started to revive. After the incubation

period, the inoculum concentrations were reduced by several-fold compared to the control for the bacteria treated with gallic acid and hamamelitannin alone or in combination with ceftiofur, erythromycin or ampicillin, indicating that these phenolic compounds alone and in combination with ceftiofur, erythromycin and ampicillin have bacteriostatic effects.

To investigate the antibacterial mode of action, it is essential to evaluate changes in the bacterial cell membrane permeability, integrity, morphology and surface characteristics [52, 53]. The physiological and morphological changes in *E. coli* and *S.* Typhimurium were observed by SEM after treatment with gallic acid and hamamelitannin alone and in combination with ceftiofur, erythromycin and ampicillin. The results showed a direct effect of the combination antibacterials on the tested pathogens. The treated bacterial cells showed obvious morphological changes compared to untreated cells. Almost all treated bacterial cells were found as long and rope-like, with no binary fission, which is completely different from the untreated control cells. These observations indicated that the combination antibacterials had a major effect on the bacterial cell division.

S. Typhimurium (ATCC 14028) biofilm formation in the presence of the gallic acid-ceftiofur combination and *E. coli* (ATCC 25922) biofilm formation in the presence of the hamamelitannin-erythromycin and gallic acid-ampicillin combinations were evaluated using a static biofilm assay. The inhibition of both planktonic and biofilm cells of these bacteria was induced by the combination drugs more than the individual drugs in most cases. The surviving and dead biofilm populations in the presence of the combination antibacterials were determined by imaging the BacLight live/dead-stained biofilm by CLSM. These results revealed that in addition to being bacteriostatic, the gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin combinations also appeared to act against the biofilm matrix. The large effect of the gallic acid-ceftiofur and gallic acid-ampicillin combinations against the biofilm cells of *S.* Typhimurium and *E. coli*, respectively, might be due to the small molecular size of gallic acid (170.12 g/mol), which easily penetrates into the biofilm. Subsequently, these combination antibacterials seem to destroy the biofilm matrix, resulting in the detachment of cells and thus the biofilm cells become more exposed and susceptible.

Motility is one of the pathogenic phenotypes of bacteria that contribute to the migration and dispersion of bacteria and their escape from the host immune response [37]. Flagella are known to be involved in swimming motility and play a role in biofilm formation, as well as swarming motility [49]. Recent reports mentioned that, similar to biofilms, swarming cells also show a higher degree of resistance to a variety of antibiotics [38, 55]. In this study, we investigated the ability of the gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin combinations to inhibit the swarming and swimming activities of *S.* Typhimurium and *E. coli*. The results (Figure 5) showed significant inhibition of swimming and swarming motilities with the addition of gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin combinations. The lack of swimming and swarming motilities in the presence of the combination antibacterials suggest that these agents might have some effects on flagella-related processes, namely, flagella biosynthesis, rotation, and chemotaxis, which may lead to decreased swimming and swarming activities.

The evaluation of the safety/toxicity profiles of any drug is desirable and an essential part of the investigation of the pharmacological effects. Moreover, humans are very often exposed to ingested contaminants or toxins. Virtually all organs and tissues are exposed, once the ingested toxins cross the intestinal wall [56]. Hence, the cytotoxic effects of gallic acid and hamamelitannin alone, and gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin combinations were investigated in Hep G2 and IEC-6 cell lines. The IC_{50} values of gallic acid and hamamelitannin were 1427.34 µM and 2024.97 µM in Hep G2 cells, and 564.55 µM and 988.54 µM in IEC-6 cells, respectively. The effects of gallic acid and hamamelitannin on cell viability were evaluated previously with different cell lines and found to have cytoprotective effects or no adverse effects in cell viability [57, 58]. Moreover, the IC_{50} values of gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin combinations obtained in this study are logical and reliable.

Conclusion

Together with all the promising *in vitro* assay findings, it is concluded that gallic acid and hamamelitannin alone, and in combination with commercial antibiotics can be promising, potent and noble candidates to eradicate pathogenic *S.* Typhimurium and *E. coli.* The effects of gallic acid and hamamelitannin are bacteriostatic, and the use of these 2 phenolic compounds in combination with commercial antibiotics can more effectively interfere with the biofilm than the antibiotics alone, which is crucial to develop new antimicrobials and/or improve the efficacy of existing antimicrobials to reduce the pathogenicity associated with these bacteria. All the findings of this study suggest that gallic acid and hamamelitannin can serve as potential medications to treat *S.* Typhimurium and *E. coli* infections in the future. Further study is recommended to determine the specific mechanisms of the synergistic antibacterial effects of these compounds and to confirm their efficacy in *in vivo* systems. It is also suggested to reveal the safety profile of these antibacterial combinations both in *in vitro* and *in vivo* system for enhancing their safe and effective utilization.

Abbreviations

AMP: Ampicillin; CDC: Canter for disease control and prevention; CEF: Ceftiofur; CLSI: Clinical and laboratory standard institute; CLSM: Confocal laser scanning microscope; DDW: Double-distilled water; DMEM: Dulbecco's modified eagle's medium; EMEM: Eagle's minimum essential medium; ERY: Erythromycin; FBS: Foetal bovine serum; FIC: Fractional inhibitory concentration; FICI: Fractional inhibitory concentration index; GA: Gallic acid; GIC_{50}: 50% growth inhibition concentration; HAMA: Hamamelitannin; IC_{50}: Inhibitory concentration 50%; MDR: Multidrug-resistant; MHA: Mueller hinton agar; MHB: Mueller hinton broth; MIC: Minimum inhibitory concentration; NB: Nutrient broth; NTME: *Nymphaea tetragona* 50% methanol extract; OD: Optical density; PBS: Phosphate buffer saline; QC: Quality control; SEM: Scanning electronic microscope; TSB: Trypticase soy broth; WHO: World health organization.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by Veterinary Science Research Project grants from the Korean Animal and Plant Quarantine Agency (Grant no. B-1543073-2017-17-0101). The funding body has no role in the design of the study and collection, analysis, and interpretation of data, and in writing the manuscript.

Authors' contributions

MAH conceived the study, and involved in conception, design, acquisition of data, analysis, interpretation, drafting and critically revising the manuscript; JWK and HCP were involved in some of the experiments and reviewed the manuscript; JWK, SUP and KJL analyzed the data and approved the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank all members of the laboratory of antibiotics and therapeutics, Veterinary Drugs & Biologics Division, Animal and Plant Quarantine Agency for their support during this study.

Authors' information

[1]Veterinary Drugs & Biologics Division, Animal and Plant Quarantine Agency, Gimcheon-si 39660, Republic of Korea. [2]Laboratory of Veterinary Pharmacokinetics and Pharmacodynamics, College of Veterinary Medicine, Kyungpook National University, Bukgu, Daegu 41566, Republic of Korea.

References

1. Fact sheet: the top 10 causes of death. World Health Organization, Geneva. 2019. http://www.who.int/mediacentre/factsheets/fs310/en/index2.html. Accessed 26 Mar 2019.

2. Farooqui A, Khan A, Borghetto I, Kazmi SU, Rubino S, Paglietti B. Synergistic antimicrobial activity of *Camellia sinensis* and *Juglans regia* against multidrug-resistant bacteria. PLoS One. 2015;10(2):e0118431. doi: 10.1371/journal.pone.0118431

3. Golkar Z, Bagasra O, Pace DG. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. J Infect Dev Ctries. 2014;8(2):129−36. doi: 10.3855/jidc.3573

4. Gould IM, Bal AM. New antibiotic agents in the pipeline and how they can help overcome microbial resistance. Virulence. 2013;4(2):185−91. doi: 10.4161/viru.22507

5. Wright GD. Something old, something new: revisiting natural products in antibiotic drug discovery. Can J Microbiol. 2014;60(3):147−54. doi: 10.1139/cjm-2014-0063

6. Sengupta S, Chattopadhyay MK, Grossart HP. The multifaceted roles of antibiotics and antibiotic resistance in nature. Front Microbiol. 2013;4:47. doi: 10.3389/fmicb.2013.00047

7. Antibiotic Resistance Threats in the United States. Centers for Disease Control and Prevention, U.S. Department of Health and Human Services. 2013. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf. Accessed 26 Mar 2019

8. Shrestha L. Life expectancy in the United States. Congressional research service report. 2005. https://www.research.policyarchive.org/2355.pdf. Accessed 26 Mar 2019

9. Viswanathan VK. Off-label abuse of antibiotics by bacteria. Gut Microbes. 2014;5(1):3−4. doi: 10.4161/gmic.28027

10. Read AF, Woods RJ. Antibiotic resistance management. Evol Med Public Health. 2014;2014(1):147. doi: 10.1093/emph/eou024

11. The antibiotic alarm. Nature. 2013;495(7440):141. doi: 10.1038/495141a

12. Lushniak BD. Antibiotic resistance: a public health crisis. Public Health Rep. 2014;129(4):314−6. doi: 10.1177/003335491412900402

13. Gross M. Antibiotics in crisis. Curr Biol. 2013;23(24):R1063−5. doi: 10.1016/j.cub.2013.11.057

14. Piddock LJ. The crisis of no new antibiotics–what is the way forward? Lancet Infect Dis. 2012;12(3):249−53. doi: $1016/S1473-3099(11)70316-4$

15. Bartlett JG, Gilbert DN, Spellberg B. Seven ways to preserve the miracle of antibiotics. Clin Infect Dis. 2013;56(10):1445−50. doi: 10.1093/cid/cit070

16. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. Front Public Health. 2014;2:145. doi: 10.3389/fpubh.2014.00145

17. Rossolini GM, Arena F, Pecile P, Pollini S. Update on the antibiotic resistance crisis. Curr Opin Pharmacol. 2014;18:56−60. doi: 10.1016/j.coph.2014.09.006

18. Tacconelli E, Magrini N. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. World Health Organization, Geneva. 2017. https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1. Accessed 26 Mar 2019

19. Courtney CM, Goodman SM, Nagy TA, Levy M, Bhusal P, Madinger NE, Detweiler CS, Nagpal P, Chatterjee A. Potentiating antibiotics in drug-resistant clinical isolates via stimuli-activated superoxide

generation. Sci Adv. 2017;3(10):e1701776. doi: 10.1126/sciadv.1701776

20. Patra JK, Baek KH. Antibacterial activity and synergistic antibacterial potential of biosynthesized silver nanoparticles against foodborne pathogenic bacteria along with its anticandidal and antioxidant effects. Front Microbiol. 2017;8:167. doi: 10.3389/fmicb.2017.00167

21. Acharyya S, Sarkar P, Saha DR, Patra A, Ramamurthy T, Bag PK. Intracellular and membrane-damaging activities of methyl gallate isolated from *Terminalia chebula* against multidrug-resistant *Shigella* J Med Microbiol. 2015;64(8):901–9. doi: 10.1099/jmm.0.000107

22. Ozcan T, Akpinar-Bayizit A, Yilmaz-Ersan L, Delikanli B. Phenolics in human health. Int J Chem Eng Appl. 2014;5(5):393–6. doi: 10.7763/IJCEA.2014.V5.416

23. Hossain MA, Lee SJ, Park JY, Reza MA, Kim TH, Lee KJ, Suh JW, Park SC. Modulation of quorum sensing-controlled virulence factors by *Nymphaea tetragona* (water lily) extract. J Ethnopharmacol. 2015;174:482–91. doi: 10.1016/j.jep.2015.08.049

24. Hossain MA, Park JY, Kim JY, Suh JW, Park SC. Synergistic effect and antiquorum sensing activity of *Nymphaea tetragona* (water lily) extract. Biomed Res Int. 2014;2014:562173. doi: 1155/2014/562173

25. Pimenta ADL, Chiaradia-Delatorre LD, Mascarello A, de Oliveira KA, Leal PC, Yunes RA, de Aguiar CB, Tasca CI, Nunes RJ, Smania A Jr. Synthetic organic compounds with potential for bacterial biofilm inhibition, a path for the identification of compounds interfering with quorum sensing. Int J Antimicrob Agents 2013;42(6):519–23. doi: 10.1016/j.ijantimicag.2013.07.006

26. Hossain MA, Lee SJ, Park NH, Mechesso AF, Birhanu BT, Kang J, Reza MA, Suh JW, Park SC. Impact of phenolic compounds in the acyl homoserine lactone-mediated quorum sensing regulatory pathways. Sci Rep. 2017;7:10618. doi: 10.1038/s41598-017-10997-5

27. Birhanu BT, Park NH, Lee SJ, Hossain MA, Park SC. Inhibition of *Salmonella* Typhimurium adhesion, invasion, and intracellular survival via treatment with methyl gallate alone and in combination with marbofloxacin. Vet Res. 2018;49(1):101. doi: 10.1186/s13567-018-0597-8

28. Yong YC, Zhong JJ. Impacts of quorum sensing on microbial metabolism and human health. Adv Biochem Eng Biotechnol. 2013;131:25–61. doi: 10.1007/10_2012_138

29. Wang Y. The anti-oxidation and anti-microbial activities of tea polyphenols and its increased reagents. J Biol. 2007;24:54–6.

30. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. CLSI Approved Standards M7-A5. Clinical and Laboratory Standards Institute, Wayne. 2000. https://clsi.org/media/1928/m07ed11_sample.pdf. Accessed 26 Mar 2019.

31. Reddy VM, Einck L, Nacy CA. *In vitro* antimycobacterial activities of capuramycin analogues. Antimicrob Agents Chemother. 2008;52(2):719–21. doi: 1128/AAC.01469-07

32. Shalit I, Shadkchan Y, Samra Z, Osherov N. *In vitro* synergy of caspofungin and itraconazole against *Aspergillus*: MIC versus minimal effective concentration end points. Antimicrob Agents Chemother. 2003;47(4):1416–8. **doi:**10.1128/AAC.47.4.1416-1418.2003

33. Murtey M, Ramasamy P. Sample preparations for scanning electron microscopy–life sciences. In: Janecek M, Kral R, editors. Modern Electron Microscopy in Physical and Life Sciences. IntechOpen.

London, United Kingdom, 2016. P. 161–85.

34. McGrath DM, Barbu EM, Driessen WH, Lasco TM, Tarrand JJ, Okhuysen PC, Kontoyiannis DP, Sidman RL, Pasqualini R, Arap W. Mechanism of action and initial evaluation of a membrane active all-D-enantiomer antimicrobial peptidomimetic. Proc Natl Acad Sci USA. 2013;110(9):3477–82. doi: 10.1073/pnas.1221924110

35. Zhao T, Liu Y. N-acetylcysteine inhibit biofilms produced by *Pseudomonas aeruginosa*. BMC Microbiol 2010;10:140. doi: 10.1186/1471-2180-10-140

36. Periasamy S, Joo HS, Duong AC, Bach TH, Tan VY, Chatterjee SS, Cheung GY, Otto M. How *Staphylococcus aureus* biofilms develop their characteristic structure. Proc Natl Acad Sci USA. 2012;109(4):1281–6. doi: 10.1073/pnas.1115006109

37. Zhuang Y, Chen W, Yao F, Huang Y, Zhou S, Li H, Zhang Z, Cai C, Gao Y, Peng Q. Short-term pretreatment of sub-inhibitory concentrations of gentamycin inhibits the swarming motility of *Escherichia coli* by down-regulating the succinate dehydrogenase gene. Cell Physiol Biochem. 2016;39(4):1307–16. doi: 10.1159/000447835

38. Kim W, Killam T, Sood V, Surette MG. Swarm-cell differentiation in *Salmonella enterica* serovar Typhimurium results in elevated resistance to multiple antibiotics. J Bacteriol. 2003;185(10):3111–7. doi: 10.1128/JB.185.10.3111-3117.2003

39. Lai LH, Fu QH, Liu Y, Jiang K, Guo QM, Chen QY, Yan B, Wang QQ, Shen JG. Piperine suppresses tumor growth and metastasis *in vitro* and *in vivo* in a 4T1 murine breast cancer model. Acta Pharmacol Sin. 2012;33(4):523–30. doi: 10.1038/aps.2011.209

40. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Testing for Bacteria Isolated From Animals. CLSI Approved Standards M31-A3. Clinical and Laboratory Standards Institute, Wayne. 2008. https://www.dbt.univr.it/documenti/OccorrenzaIns/matdid/matdid485539.pdf. Accessed 26 Mar 2019.

41. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0. European Committee on Antimicrobial Susceptibility Testing, Basel. 2019. http://www.eucast.org/clinical_breakpoints/. Accessed 26 Mar 2019.

42. Sanhueza L, Melo R, Montero R, Maisey K, Mendoza L, Wilkens M. Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against *Staphylococcus aureus* and *Escherichia coli*. PLoS ONE. 2017;12(2):e0172273. doi: 10.1371/journal.pone.0172273

43. Clinical Microbiology and Infection. Statement 1996 CA-SFM Zone sizes and MIC breakpoints for non-fastidious organisms. Clin Microbiol Infect. 1996;2 Suppl 1: S46–9. doi: 10.1111/j.1469-0691.1996.tb00875.x

44. Wei CF, Chang SK, Shien JH, Kuo HC, Chen WY, Chou CC. Synergism between two amphenicol of antibiotics, florfenicol and thiamphenicol, against *Staphylococcus aureus*. Vet Rec.2016;178(13): doi: 10.1136/vr.103554

45. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74(3):417–33. doi: 10.1128/MMBR.00016-10

46. Berahou A, Auhmani A, Fdil N, Benharref A, Jana M, Gadhi CA. Antibacterial activity of *Quercus ilex* bark's extracts. J Ethnopharmacol. 2007;112(3):426–9. doi: 1016/j.jep.2007.03.032

47. Salomao K, Pereira PR, Campos LC, Borba CM, Cabello PH, Marcucci MC, De Castro SL. Brazilian propolis: correlation between chemical composition and antimicrobial activity. Evid Based Complement Alternat Med. 2008;5(3):317–24. doi: 10.1093/ecam/nem058

48. Fu L, Lu W, Zhou X. Phenolic compounds and *in vitro* antibacterial and antioxidant activities of three tropic fruits: persimmon, guava, and sweetsop. BioMed Res Int. 2016;2016:4287461. doi: 1155/2016/4287461

49. Taguri T, Tanaka T, Kouno I. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. Biol Pharm Bull. 2004;27:1965–9. doi: 1248/bpb.27.1965

50. Dubuisson T, Bogatcheva E, Krishnan MY, Collins MT, Einck L, Nacy CA, Reddy VM. *In vitro* antimicrobial activities of capuramycin analogues against non-tuberculous mycobacteria. J Antimicrob Chemother. 2010;65(12):2590–7. doi: 10.1093/jac/dkq372

51. Pfaller MA, Sheehan DJ, Rex JH. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. Clin Microbiol Rev. 2004;17(2):268–80. doi: 10.1128/CMR.17.2.268-280.2004

52. Diao W-R, Hu Q-P, Zhang H, Xu J-G. Chemical composition, antibacterial activity and mechanism of action of essential oil from seeds of fennel (*Foeniculum vulgare Mill.*). Food Control. 2014;35(1):109–16. doi: 10.1016/j.foodcont.2013.06.056

53. Sadiq MB, Tarning J, Aye Cho TZ, Anal AK. Antibacterial activities and possible modes of action of *Acacia nilotica* (L.) *Del.* against multidrug-resistant *Escherichia coli* and *Salmonella*. Molecules. 2017;22(1):47. doi: 10.3390/molecules22010047

54. de la Fuente-Nunez C, Korolik V, Bains M, Nguyen U, Breidenstein EB, Horsman S, Lewenza S, Burrows L, Hancock RE. Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. Antimicrob Agents Chemother. 2012;56(5):2696–704. **doi:**1128/AAC.00064-12

55. Lai S, Tremblay J, Deziel E. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. Environ Microbiol. 2009;11(1):126–36. doi: 10.1111/j.1462-2920.2008.01747.x

56. Grenier B, Applegate TJ. Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals. Toxins. 2013;5(2):396–430. doi: 10.3390/toxins5020396

57. Yang DJ, Moh SH, Son DH, You S, Kinyua AW, Ko CM, Song M, Yeo J, Choi YH, Kim KW. Gallic acid promotes wound healing in normal and hyperglucidic conditions. Molecules. 2016;21(7):E899. doi: 10.3390/molecules21070899

58. Masaki H, Atsumi T, Sakurai H. Protective activity of hamamelitannin on cell damage of murine skin fibroblasts induced by UVB irradiation. J Dermatol Sci. 1995;10(1):25–34. doi: 10.1016/0923-1811(95)93711-9

Tables

Table 1: Minimum inhibition concentration of commercial antibiotics and phenolic compounds against different strains; and antibacterial sensitivity profiles of *Salmonella enterica serovar* Typhimurium and *Escherichia coli* against some selected commercially available antibiotics.

Antimicrobials		*Salmonella enterica serovar* Typhimurium						*Escherichia coli*
		ATCC 14028	V08-S-HA-06-(170)	V15-S-HA-02-(210)	SAL 109	SAL 202	SAL 224	ATCC 25922
Amoxicillin	MIC (µg/mL)	<0.5	256	1	256	256	256	1
	Sensitivity	S	R	S	R	R	R	S
Ampicillin	MIC (µg/mL)	1	512	512	512	512	512	2
	Sensitivity	S	R	R	R	R	R	S
Cefotaxime	MIC (µg/mL)	≤2	≤2	≤2	512	128	128	0.125
	Sensitivity	S	S	S	R	R	R	S
Ceftiofur	MIC (µg/mL)	<1	<1	1	128	64	64	1
	Sensitivity	S	S	S	R	R	R	S
Erythromycin	MIC (µg/mL)	128	32	16	512	1024	512	128
	Sensitivity	R	R	R	R	R	R	R
Florfenicol	MIC (µg/mL)	8	8	4	64	32	64	8
	Sensitivity	S	S	S	R	R	R	S
Marbofloxacin	MIC (µg/mL)	0.062	0.25	0.25	2	2	4	0.25
	Sensitivity	S	S	S	I	I	I	S
Norfloxacin	MIC (µg/mL)	0.25	4	0.5	4	16	8	0.5
	Sensitivity	S	R	S	R	R	R	S

Penicillin G	MIC (µg/mL)	8	1024	32	>1024	>1024	>1024	16
	Sensitivity	S	R	R	R	R	R	R
Thiamphenicol	MIC (µg/mL)	128	64	128	256	512	512	256
	Sensitivity	R	R	R	R	R	R	R
Epicatechin	MIC (µg/mL)	>1024	>1024	>1024	>1024	>1024	>1024	≥1024
Epicatechin gallate	MIC (µg/mL)	>512	512	>512	>512	>512	>512	1024
Epigallocatechin	MIC (µg/mL)	1024	512	512	512	512	512	512
Gallic acid	MIC (µg/mL)	256	256	256	256	256	256	1024
Hamamelitannin	MIC (µg/mL)	512	512	1024	1024	1024	1024	2048

MIC, minimum inhibition concentration; S, susceptible; I, intermediate resistance; R, resistant. Sensitivity statuses of these strains are interpreted based on the MIC values of these tested antibiotics and their break-point MIC values mentioned in different guidelines [40–44].

Table 2: Combination interaction of phenolic compounds with commercial antibiotics against *Salmonella enterica serovar* Typhimurium (ATCC14028) and *Escherichia coli* (ATCC25922).

Strains	Phenolic Compounds (Drug A)	Antibiotics (Drug B)	FIC Index (X)	Interpretations
S. Typhimurium	Galic Acid	Ampicillin	0.625	Additive
		Amoxicillin	1.004	Indifferent
		Ceftiofur	0.563	Additive
		Penicillin G	1.016	Indifferent
		Cefotaxime	1.004	Indifferent
		Erythromycin	1.001	Indifferent
		Thiamphenicol	0.625	Additive
		Marbofloxacin	1.002	Indifferent
	Hamamelitannin	Ampicillin	1.002	Indifferent
		Amoxicillin	1.001	Indifferent
		Ceftiofur	0.625	Additive
		Penicillin G	1.004	Indifferent
		Cefotaxime	0.563	Additive
		Erythromycin	1.001	Indifferent
		Thiamphenicol	1.002	Indifferent
		Marbofloxacin	0.563	Additive
	Epicatechin	Ampicillin	0.750	Additive
		Amoxicillin	1.001	Indifferent
		Ceftiofur	0.625	Additive
		Penicillin G	1.004	Indifferent
		Cefotaxime	0.750	Additive
		Erythromycin	1.016	Indifferent
		Thiamphenicol	1.016	Indifferent
		Marbofloxacin	1.004	Indifferent
	Epicatechin Gallate	Ampicillin	0.563	Additive
		Amoxicillin	1.002	Indifferent
		Ceftiofur	1.001	Indifferent

		Penicillin G	0.502	Additive
		Cefotaxime	1.004	Indifferent
		Erythromycin	0.500	Synergy
		Thiamphenicol	0.504	Additive
		Marbofloxacin	1.008	Indifferent
	Epigallocatechin	Ampicillin	1.001	Indifferent
		Amoxicillin	1.004	Indifferent
		Ceftiofur	0.625	Additive
		Penicillin G	1.008	Indifferent
		Cefotaxime	0.502	Additive
		Erythromycin	0.516	Additive
		Thiamphenicol	1.004	Indifferent
		Marbofloxacin	0.502	Additive
E. coli	Galic Acid	Ampicillin	0.504	Additive
		Amoxicillin	1.008	Indifferent
		Ceftiofur	1.001	Indifferent
		Penicillin G	1.004	Indifferent
		Cefotaxime	0.563	Additive
		Erythromycin	1.002	Indifferent
		Thiamphenicol	0.281	Synergy
		Marbofloxacin	0.625	Additive
	Hamamelitannin	Ampicillin	1.001	Indifferent
		Amoxicillin	0.625	Additive
		Ceftiofur	1.002	Indifferent
		Penicillin G	1.002	Indifferent
		Cefotaxime	1.001	Indifferent
		Erythromycin	0.375	Synergy
		Thiamphenicol	0.500	Synergy
		Marbofloxacin	0.625	Additive
	Epicatechin	Ampicillin	1.004	Indifferent

	Amoxicillin	1.001	Indifferent
	Ceftiofur	0.625	Additive
	Penicillin G	1.002	Indifferent
	Cefotaxime	0.563	Additive
	Erythromycin	0.504	Additive
	Thiamphenicol	1.008	Indifferent
	Marbofloxacin	0.504	Additive
Epicatechin Gallate	Ampicillin	1.004	Indifferent
	Amoxicillin	1.001	Indifferent
	Ceftiofur	0.625	Additive
	Penicillin G	1.002	Indifferent
	Cefotaxime	1.002	Indifferent
	Erythromycin	1.001	Indifferent
	Thiamphenicol	0.625	Additive
	Marbofloxacin	0.516	Additive
Epigallocatechin	Ampicillin	0.504	Additive
	Amoxicillin	1.008	Indifferent
	Ceftiofur	0.625	Additive
	Penicillin G	1.008	Indifferent
	Cefotaxime	0.502	Additive
	Erythromycin	0.516	Additive
	Thiamphenicol	1.008	Indifferent
	Marbofloxacin	0.504	Additive

FIC, fractional inhibitory concentration; Synergy, $X \leq 0.5$; Additive, $0.5 < X \leq 1$; Indifferent, $1 < X \leq 2$; Antagonist, $X > 2$.

Table 3: Effect of ceftiofur-gallic acid combination on the swimming and swarming motilities of *Salmonella enterica serovar* Typhimurium (ATCC14028), and erythromycin-

hamamelitannin and ampicillin-gallic acid combination on the swimming and swarming motilities of *Escherichia coli* (ATCC25922).

Strains	Treatment Groups	Swarming (mm) (mean±SD)	Swimming (mm) (mean±SD)
Salmonella enterica serovar Typhimurium	Control	9.67±2.08[a]	26.67±2.52[a]
	Ceftiofur MIC	2.00±1.00[d]	5.33±2.31[c]
	Gallic acid MIC	6.33±1.15[b]	7.00±1.00[b]
	Ceftiofur MIC + Gallic acid MIC	1.33±0.58[e]	0.00±0.00
	Ceftiofur ⯪MIC + Gallic acid ⯪MIC	3.33±1.15[c]	2.33±1.53[d]
Escherichia coli	Control	23.33±2.08[a]	22.00±1.73[a]
	Erythromycin MIC	6.67±1.15[b]	5.67±0.58[c]
	Hamamelitannin MIC	3.33±0.58[c]	7.33±1.53[b]
	Erythromycin MIC + Hamamelitannin MIC	0.00±0.00	0.00±0.00
	Erythromycin ⯪MIC + Hamamelitannin ⯪MIC	0.00±0.00	2.67±0.58[d]
Escherichia coli	Control	21.67±2.52[a]	21.33±3.06[a]
	Ampicillin MIC	11.33±1.53[b]	5.67±1.15[b]
	Gallic acid MIC	4.00±1.73[c]	4.33±1.53[c]
	Ampicillin MIC + Gallic acid MIC	0.00±0.00	1.67±1.53[d]
	Ampicillin ⯪MIC + Gallic acid ⯪MIC	3.67±1.15[c]	4.00±1.00[c]

MIC: minimum inhibitory concentration. Different superscript letters indicate statistical significance ($P< 0.05$).

Table 4: Effects of gallic acid and hamanelitannin alone, and in combination with ampicillin, ceftiofur and erythromycin on the viability of human hepatocyte (Hep G2) and *Rattus norvegicus* small intestine (IEC-6) cells.

Antibacterials	Inhibitory Concentration 50% (µM)	
	Hep G2	IEC-6
Ampicillin	12694.59	2556.66
Ceftiofur	15587.93	694.05
Erythromycin	14330.33	515.50
Gallic acid	1427.34	564.55
Hamamelitannin	2024.97	988.54
Gallic acid (in presence of Ampicillin)	10476.52	1746.86
Ampicillin (in presence of Gallic acid)	5020	828.90
Gallic acid (in presence of Ceftiofur)	11407.11	1907.55
Ceftiofur (in presence of Gallic acid)	7106	604.83
Hamamelitannin (in presence of Erythromycin)	8452.73	564.55
Erythromycin (in presence of Hamamelitannin)	5469.41	777.09

Additional File

Additional file 1: Effects of (A) gallic acid and (B) hamanelitannin on the viability of *Rattus norvegicus* small intestine (IEC-6) cells. Effects of (C) gallic acid and (D) hamanelitannin on the viability of human hepatocyte (Hep G2). Results are interpreted from 3 independent experiments.

Figures
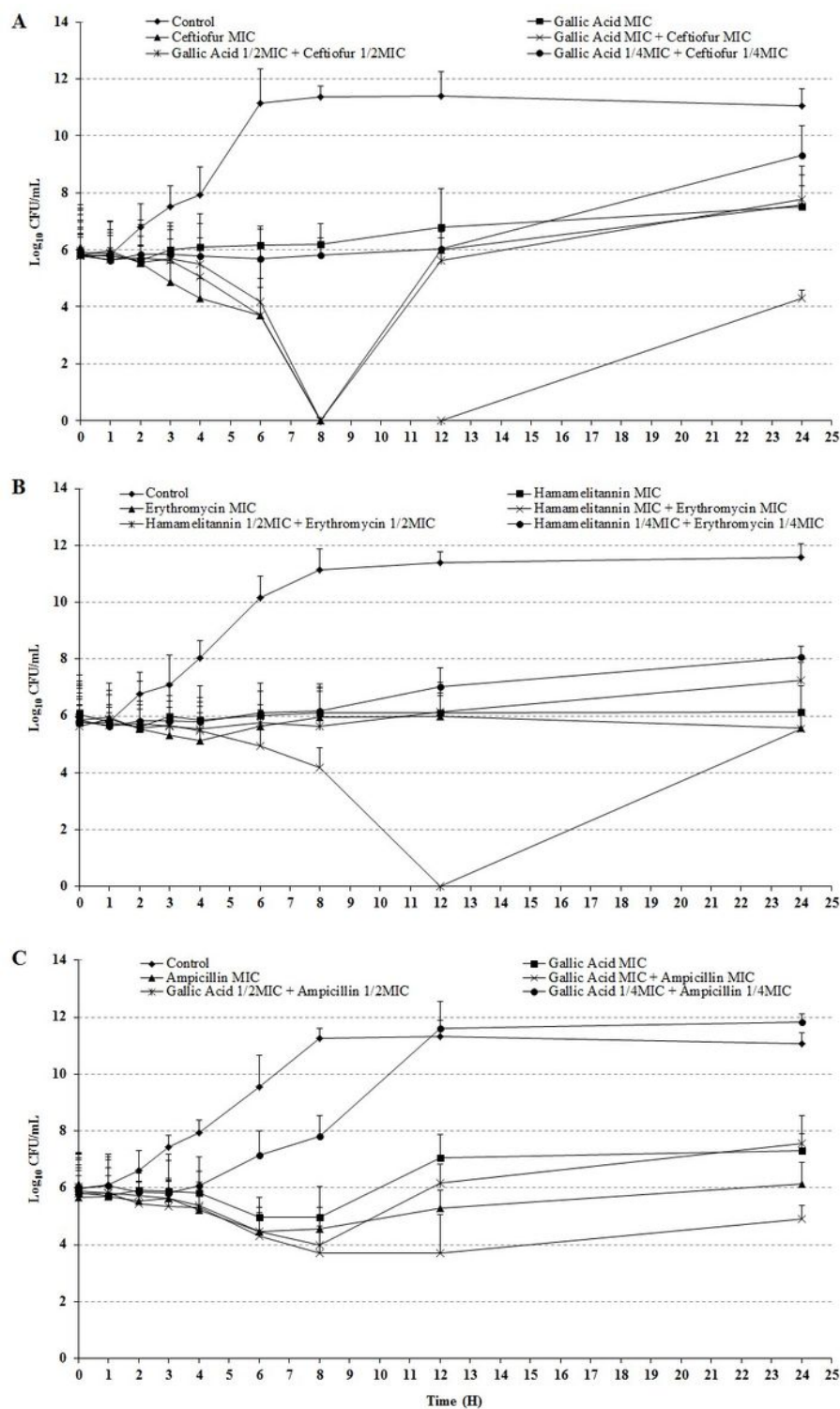
Figure 1

Time-kill curves of (A) Salmonella enterica serovar Typhimurium (ATCC14028), (B) Escherichia coli (ATCC25922), and (C) Escherichia coli (ATCC25922) in presence of gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin combinations, respectively. MIC, minimum inhibitory concentration.
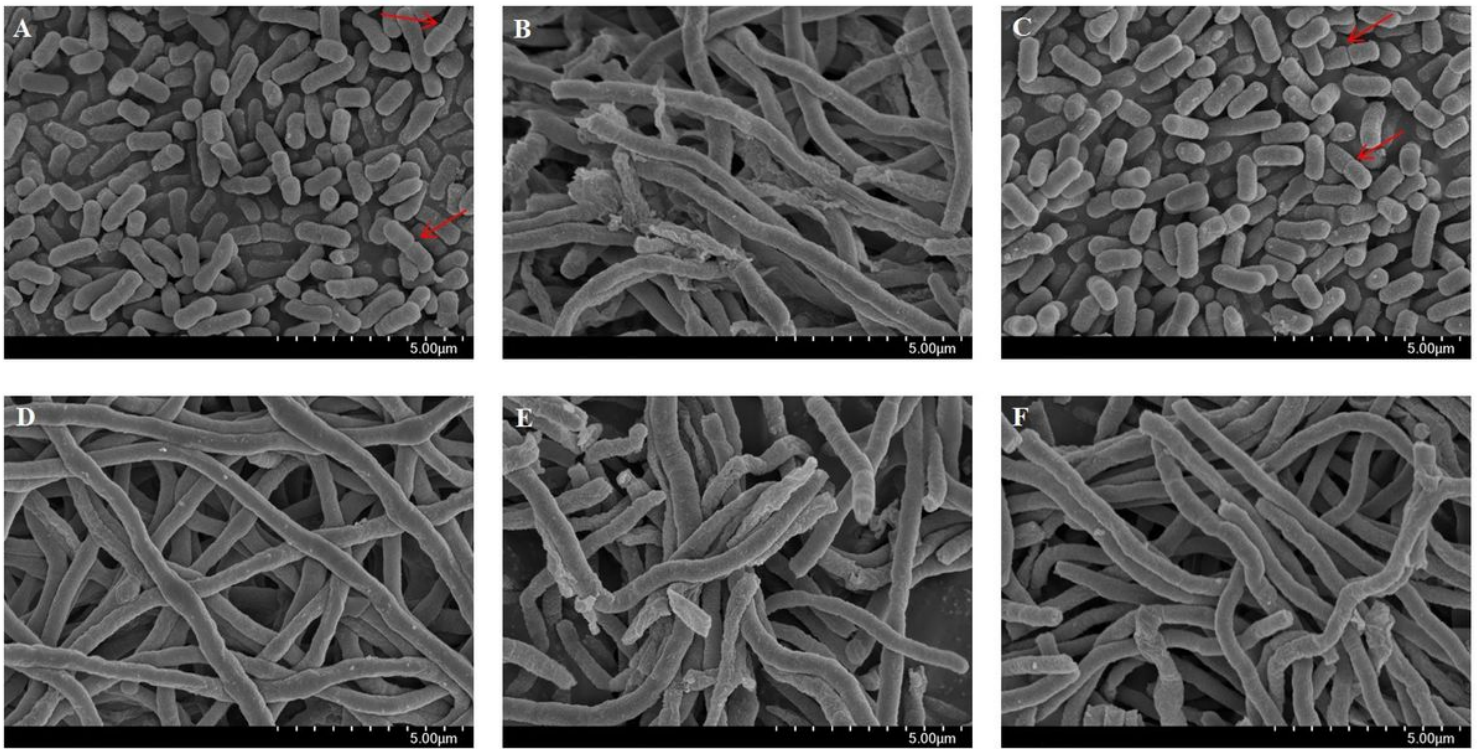
Figure 2

Effect of antibacterial combination on the ultrastructure morphology of bacterial cells. Representative images of Salmonella enterica serovar Typhimurium (ATCC14028) cells captured by scanning electron microscope after treatment with gallic acid and ceftiofur. Salmonella enterica serovar Typhimurium cells treated with (A) no drug, (B) 1MIC of ceftiofur, (C) 1MIC of gallic acid, (D) 1MIC of ceftiofur and 1MIC of gallic acid, (E) ½MIC of ceftiofur and ½MIC of gallic acid, and (F) ¼MIC of ceftiofur and ¼MIC of gallic acid. MIC, minimum inhibitory concentration. Arrows indicate binary fission.
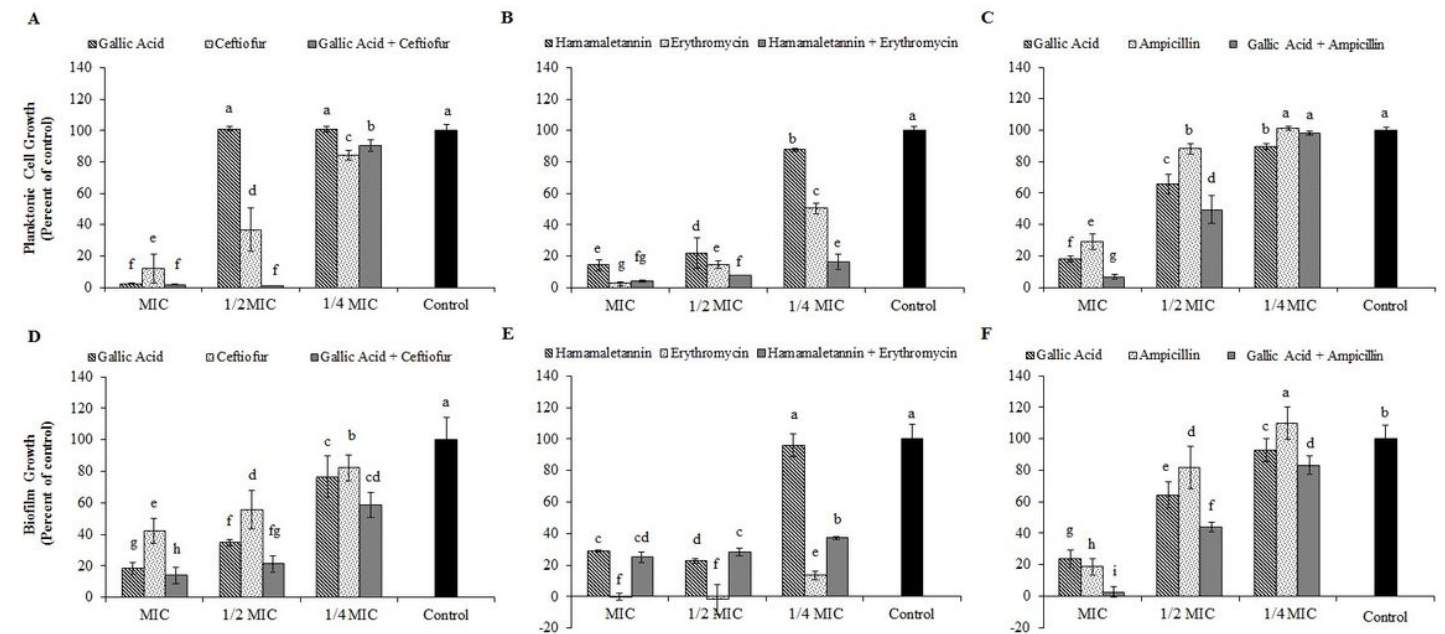
Figure 3

Effect of antibacterial combination on the on the growth of planktonic cells (upper panel) and biofilm cells (lower panel). Effect of gallic acid-ceftiofur combination on (A) planktonic and (D) biofilm cells of Salmonella enterica serovar Typhimurium (ATCC14028). Effects of hamamelitannin-erythromycin combination on (B) planktonic and (E) biofilm cells, and gallic acid-ampicillin combination on (C) planktonic and (F) biofilm cells of Escherichia coli (ATCC25922). MIC: minimum inhibitory concentration. Different superscript letters indicate statistical significance (P< 0.05).
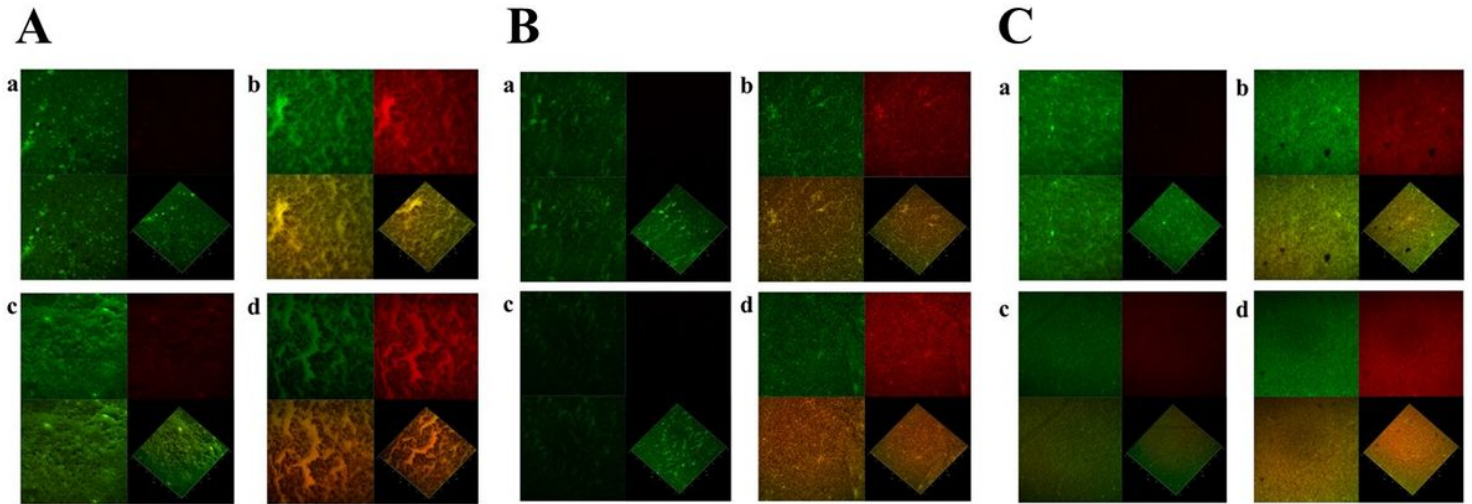
Figure 4

Effect of antibacterial combination on the viability of cultured biofilm. The confocal laser scanning microscope images of BacLight LIVE/DEAD stained biofilms of (A) Salmonella enterica serovar Typhimurium (ATCC14028) treated with [(a) no drug, (b) ½MIC of gallic acid, (c) ½MIC of ceftiofur, (d) ½MIC of gallic acid and ½MIC of ceftiofur], (B) Escherichia coli (ATCC25922) treated with [(a) no drug, (b) ½MIC of hamamelitannin, (c) ½MIC of erythromycin, (d) ½MIC of hamamelitannin and ½MIC of erythromycin], (C) Escherichia coli (ATCC25922) treated with [(a) no drug, (b) ½MIC of gallic acid, (c) ½MIC of ampicillin, (d) ½MIC of gallic acid and ½MIC of ampicillin]. The viability of the biofilm cells were assessed using BacLight LIVE/DEAD stain (green: live cells, red: dead cells). In each image, the segment at below right side shows three dimensional and other three segments show two dimensional images.
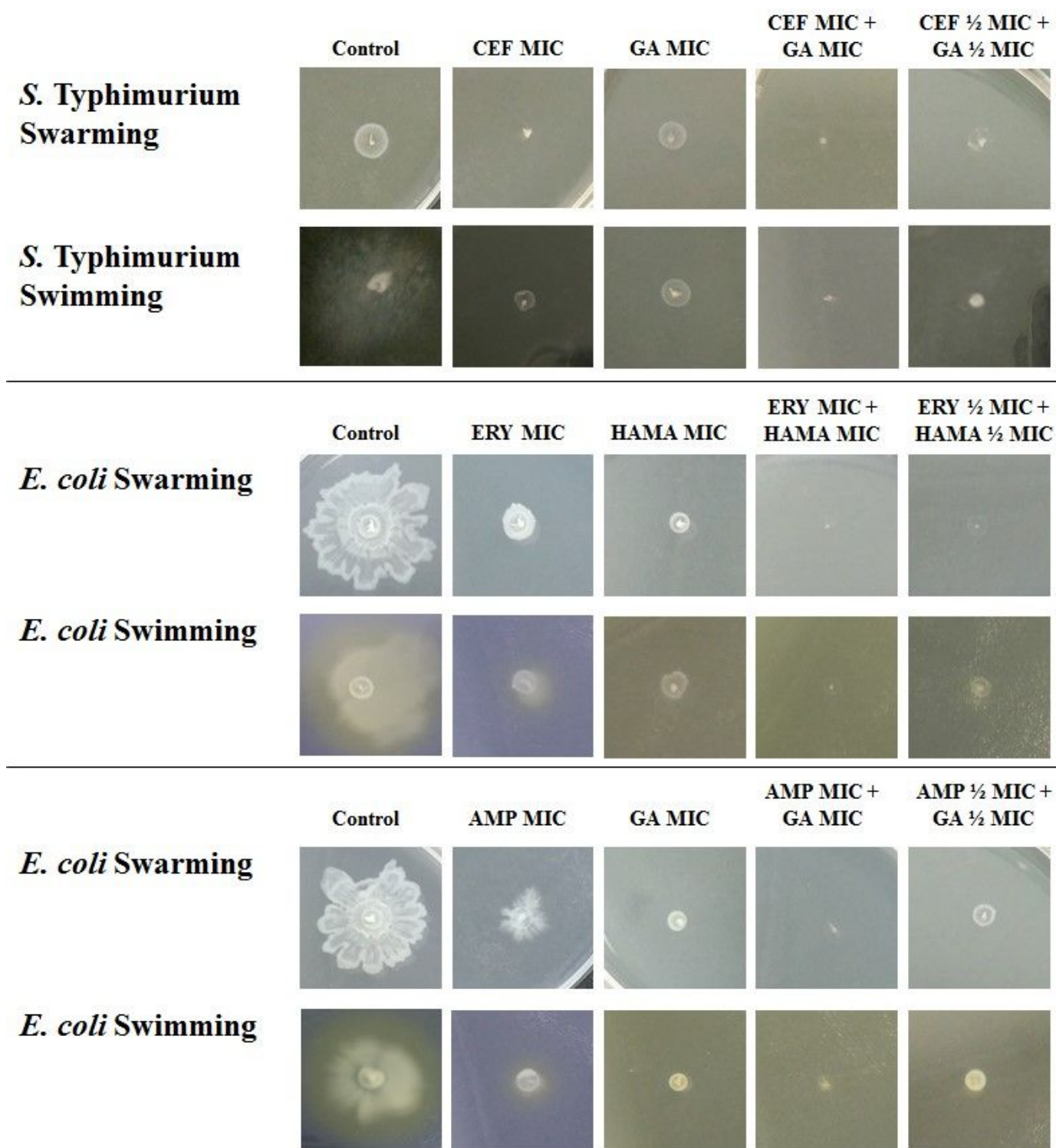
Figure 5

Representative images of swim and swarm zones of Salmonella enterica serovar Typhimurium (ATCC14028) treated with ceftiofur-gallic acid combination, and Escherichia coli (ATCC25922) treated with erythromycin-hamamelitannin and ampicillin-gallic acid combinations. CEF, ceftiofur; GA, gallic acid; ERY, erythromycin; HAMA, hamamelitannin; AMP, ampicillin; MIC: minimum inhibitory concentration.
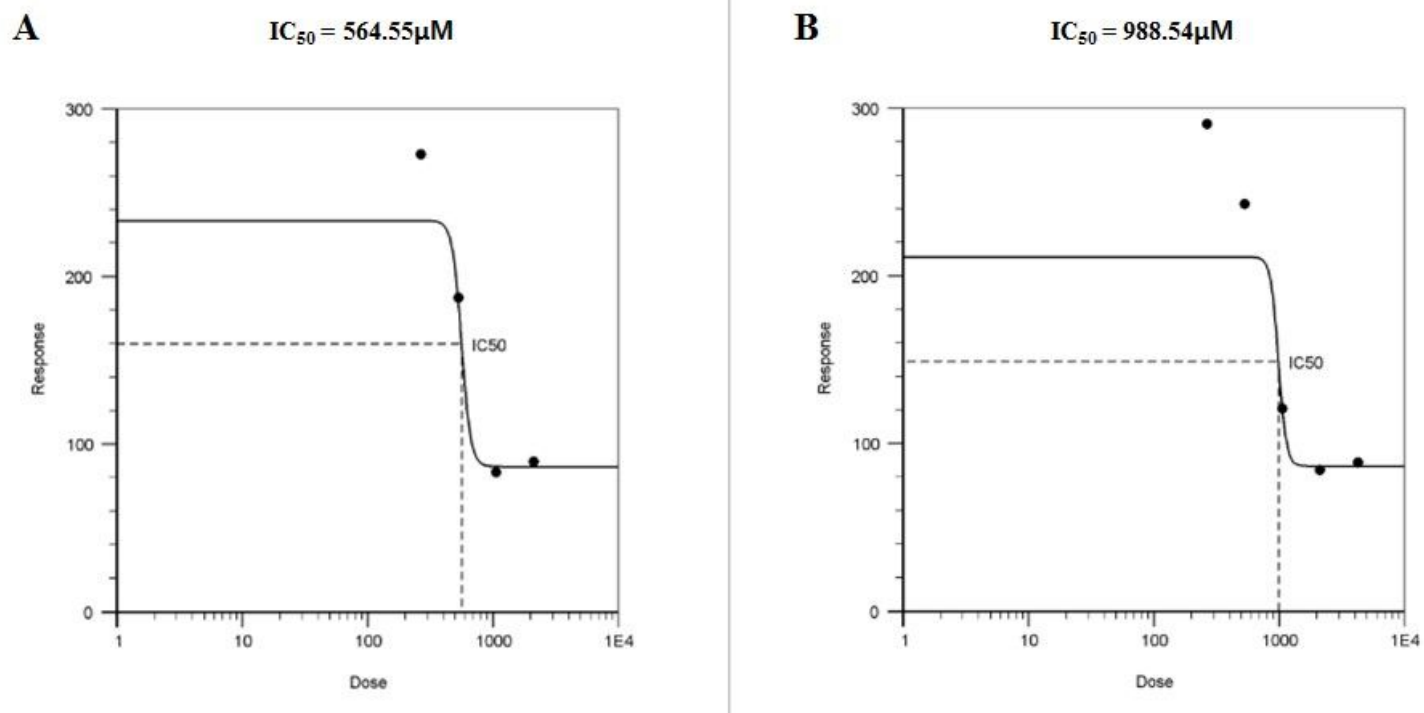
Figure 6

Effects of gallic acid and hamanelitannin on the viability of Rattus norvegicus (IEC-6) cell line. The dose-response curves of (A) gallic acid and (B) hamamelitannin.