# In vitro synergistic potentials of novel antibacterial combination therapies against Salmonella Typhimurium, Escherichia coli and Staphylococcus aureus

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# 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, Escherichia coli and Staphylococcus aureus were determined. Based on the FIC indexes 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 the Rattus norvegicus (IEC-6) cell line 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 (32–1024), (128–1024) and (512–≥2048) μg/mL, respectively. The FIC indexes of the combined antibacterials against these strains were 0.281–1.016. The ultrastructural morphology and time-kill assays 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 50% inhibitory concentrations (IC50) of GA and hamamelitannin in IEC-6 cells were 564.55 μM and 988.54 μM, respectively. Conclusions: The phenolic compounds increase the efficacy of existing antibiotics might be by disrupting virulence factors. We can conclude that these antibacterial combinations are safe and can be potential medications to treat S. Typhimurium, E. coli and S. aureus infections in animals and humans. 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, Salmonella Typhimurium and Staphylococcus aureus 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 bacterial 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 microorganism molecular targets [29]. These observations initiate the speculation that gallic acid derivatives may also potentiate the efficacy of existing antibiotics. Thus, we intended to evaluate the synergistic antibacterial potentials of gallic acid and its 4 derivatives in this study with 8 currently available antibiotics against *E. coli*, *S.* Typhimurium and S. *aureus*. Additionally, the effects of those combination antibacterials against selected virulence factors, including biofilm formation and motility, were determined. Finally, *in vitro* cytotoxicity tests of potent phenolic compounds were conducted to determine the safety profile of these compounds for further commercial use.

# Materials And Methods

Chemicals, Reagents and Bacterial Strains

Unless otherwise mentioned, all the chemicals, reagents and media were from Sigma-Aldrich (St. Louis, MO, United States). Quality control strains of *E. coli* (ATCC 25922), S. *aureus* (ATCC 25923) 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 incubated in Mueller Hinton broth (MHB; Becton Dickinson and Company, Becton Drive, NJ, United States) for 20 h in a rotating incubator at 200 rpm and 37 °C.

Minimum Inhibition Concentrations of Antibacterial Agents

MICs were determined by the standard broth microdilution method according to the CLSI guidelines [30] in cation-adjusted (CA)-MHB using an inoculum concentration of $\sim 5 \times 10^5$ 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 antibiotic 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 (FIC) 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 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 $\sim 5 \times 10^5$ 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 $\leq 0.5$ is regarded as synergistic, $0.5 < \text{FICI} \leq 1$ is considered additive, $1 < \text{FICI} \leq 2$ 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 $5 \times 10^6$ CFU/mL. A tube containing $5 \times 10^6$ 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 $5 \times 10^6$ CFU/mL. A tube containing $5 \times 10^6$ 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 Phenolic Compounds on Quorum Sensing (QS) Inhibition

The potentials of QS inhibition of phenolic compounds were verified in accordance with the method described by Alvarez et al. [34]. The standard disk diffusion assay using the biomonitor *C. violaceum* strain (ATCC 12472) was performed for this evaluation. *C. violaceum* ATCC 12472 was dispensed onto molten Luria-Bertani (LB, Becton Dickinson and Company, Becton Drive, NJ, United States) agar medium in a (90 × 15 mm) petri dish as to make the bacterial density $5 \times 10^6$ CFU/mL. Sixty microliter solutions of antibiotics that contain 60 µg of each compound were loaded onto sterile paper disks, and then the drug-containing paper disks were allowed to air dry and placed on the solidified agar. The bacteria on the agar plates were incubated overnight at 30 °C and examined for violacein production. QS inhibition was determined by the presence of a colourless, opaque, but viable halo around the disks.

Effect of Antibacterial Combinations on Biofilm Growth and Viability

The inhibitory effect of combination antibacterials on biofilm formation was determined using a slightly modified version of a previously reported spectrophotometric method [ 35 , 36 ]. Briefly, test compounds were supplemented into trypticase soy broth (TSB; Becton Dickinson and Company, Becton Drive, NJ, United States ) 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 $1 \times 10^6$ 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, 37] . 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 $1 \times 10^6$ 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 [38, 39]. The media used for the *E. coli* (ATCC 25922) swarming assay was composed of 0.8% Luria-Bertani (Becton Dickinson and Company, Becton Drive, NJ, United States) supplemented with 0.5% glucose (Scharlab, Barcelona, Spain) and 0.6% agar (Becton Dickinson and Company, Becton Drive, NJ, United States). Nutrient broth (NB; Becton Dickinson and Company, Becton Drive, NJ, United States) 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 (Scharlab, Barcelona, Spain) and 0.3% agar. Nutrient broth supplemented with 0.5% glucose and 0.25% agar was used as the media for the *S.* Typhimuriumswimming 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 a small intestine cell line of *Rattus norvegicus* (IEC-6; American Type Culture Collection CRL-1592, VA, United States) in the presence of combination drugs were evaluated according to standard EZ-cytox (EZ-1000; Daeillab Service Co. Ltd., Jeonju, South Korea) assay method. In brief, 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 foetal bovine serum (10%). The cells were subpassaged at a ratio of 1:5 twice a week. One hundred microliters of suspended cells ($2 \times 10^4$ cells/mL in the abovementioned DMEM 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 the abovementioned DMEM 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:

Cell viability (%) = (OD of drug-treated sample/OD of untreated sample) × 100, where OD is the optical density [40].

### 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*, S. *aureus*, *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*, S. *aureus* and *S.* Typhimurium ranged from 0.125~128.00 μg/mL, 0.125~4.00 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 [41–45] (Additional file 1). 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* and S. *aureus* ranged from 32.00~2048.00 μg/mL.

### *In Vitro* Synergy with Commercial Antibacterials

Checkerboard microdilution assays were performed to evaluate the combination interactions of the commercial antibacterials with the phenolic compounds. The results of the combined activities are presented in (Additional file 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), while none of the combinations showed synergistic effects against *S. aureus*. However, additive effects were obtained from many combinations against *S.* Typhimurium (FICI: 0.502~0.750; n=16), *S. aureus* (FICI: 0.504~0.625; n=13) and *E. coli* (FICI: 0.502~0.625; n=17). The rest of the combinations showed indifferent effects against the three 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 culturing 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 2 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 on the viability of *Rattus norvegicus* small intestine cells (IEC-6) were investigated. The $IC_{50}$ of gallic acid and hamamelitannin were 564.55 μM and 988.54 μM, respectively. However, the inhibitory responses of these drugs were not completely concentration-dependent within the tested concentrations.

# 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 and animals [46]. Thus, to combat infectious diseases associated with resistant pathogens, the development of alternative antimicrobial drugs is urgently needed [47, 48]. 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, *E. coli* and *S. aureus,* which have been shown to be resistant to one to eight out of ten 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, *E. coli* and *S. aureus* 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* [49]. 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 the Gram-positive bacterium *S. aureus*. The MIC value of gallic acid against the QC strain of *S. aureus* (128 μg/mL) in this study is lower than those reported previously for five strains (630 μg/mL) [49], one single strain (3200 μg/mL) [50], or 18 strains isolated from clinical cases of human impetigo and furuncle lesions (8000 μg/mL) [51], while the MIC value was higher than that reported in another study using only two strains (62.5 μg/mL) [52]. 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) [49]. The mean MICs of plant-derived epigallocatechin against *S. aureus*, *S.* Typhimurium and *E. coli* were reported to be 162±44, 572±186 and 733±121 μg/mL, respectively [53]. The MIC value of pure epigallocatechin against *S. aureus* (32 μg/mL) in our study was lower than the previously reported MIC value (162±44 μg/mL) [53]. This lower MIC value for plant-derived epigallocatechin against *S. aureus* 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. aureus*, *S.* Typhimurium and *E. coli* were 2500 μg/mL, which demonstrates the similarity between our results and previously published results [49].

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 [54]. 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), *S. aureus* (FICI: 0.504~0.625; n=13) and *E. coli* (FICI: 0.502~0.625; n=17). The rest of the combinations had indifferent effects against these three 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*, *S.* Typhimurium and/or *S. aureus*-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 [55]. 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. After the first 8h and 12 h, the bacteria treated with ½MIC and 1MIC concentrations of the combination antibacterials started their log phase, 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 [56, 57]. 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. The effects of phenolic compounds on the quorum sensing of *C. violaceum* were evaluated by agar disk diffusion assays, which demonstrated that these compounds have no noticeable anti-quorum sensing effect (data not shown).

*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 [38]. Flagella are known to be involved in swimming motility and play a role in biofilm formation, as well as swarming motility [53]. Recent reports mentioned that, similar to biofilms, swarming cells also show a higher degree of resistance to a variety of antibiotics [39, 59]. 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 3) 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. Especially considering that the combinations of gallic acid-ceftiofur, hamamelitannin-erythromycin and gallic acid-ampicillin were found to be active against intestinal bacteria. Moreover, humans are very often exposed to ingested contaminants or toxins. Although virtually all organs and tissues are exposed, once the ingested toxins cross the intestinal wall, the gut is the first organ exposed and experiences the highest concentrations/doses of toxins. Many of these toxins are able to affect intestinal functions in both animals and humans [60]. For these reasons, toxicological studies focusing on the intestine are of primary importance. Hence, the cytotoxic effects of gallic acid and hamamelitannin were investigated in a small intestine cell line of *Rattus norvegicus* (IEC-6) to determine whether these compounds have any adverse effects on intestine. The $IC_{50}$ values of gallic acid and hamamelitannin in *Rattus norvegicus* (IEC-6) cells were 564.55 μM and 988.54 μM, 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 [61, 62].

Conclusion

Together with all the promising *in vitro* assay findings, it can be concluded that gallic acid and epigallocatechin are potent and noble candidates to eradicate Gram-positive bacteria, such as *S. aureus*. Gallic acid and hamamelitannin alone and in combination with commercial antibiotics can be promising agents against Gram-negative bacteria as well, including *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 are safe and can serve as potential medications to treat *S.* Typhimurium, *E. coli* and *S. aureus* 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.

# 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; ERY: erythromycin; FIC: Fractional inhibitory concentration; FICI: FIC index; GA: gallic acid; $GIC_{50}$: 50% growth inhibition concentrations; HAMA: hamamelitannin; $IC_{50}$: 50% inhibitory concentrations; MDR: multidrug-resistant; MHA: mueller hinton agar; MHB: mueller hinton broth; MIC: minimum inhibitory concentrations; NB: nutrient broth; NTME: *Nymphaea tetragona* 50% methanol extract; OD: optical density; PBS: phosphate buffer saline; QC: quality control; QS: quorum sensing; SEM: scanning electronic microscope; TSB: trypticase soy broth; WHO: world health organization.

# Declarations

*Competing Interests*

None of the authors have any conflicts of interest to declare.

*Availability of Data and Materials*

Data will be shared upon request to the corresponding author.

*Ethics Approval and Consent to Participate*

All procedures have been approved by the Bioethical Committee of Animal and Plant Quarantine Agency, Republic of Korea.

*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.

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# Tables

**Table 1:** Minimum Inhibition Concentration of commercial antibiotics and phenolic compounds against different strains.

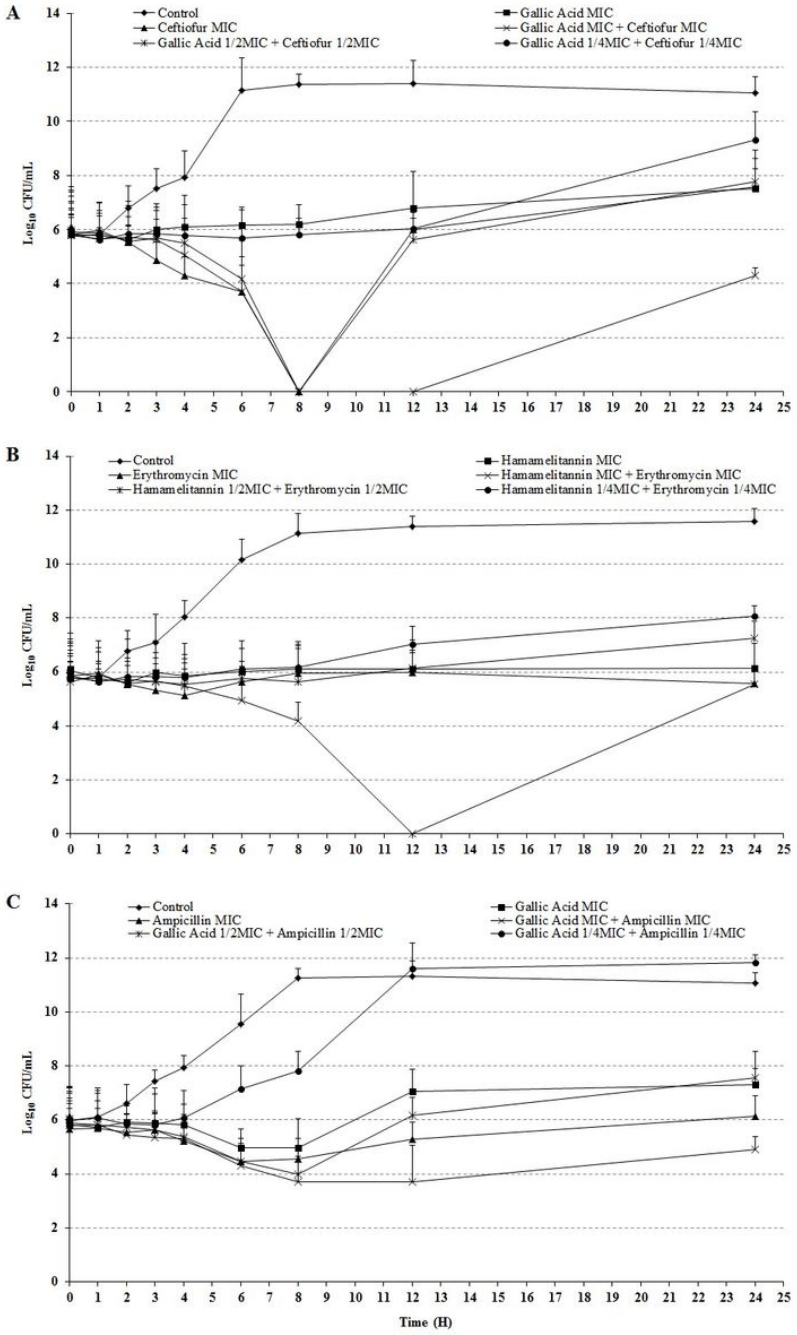
| Antimicrobials | Minimum Inhibition Concentration (MIC, µg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Salmonella enterica serovar* Typhimurium | | | | | | *Escherichia coli* | *Staphylococcus aureus* |
| | ATCC 14028 | V08-S-HA-06-(170) | V15-S-HA-02-(210) | SAL 109 | SAL 202 | SAL 224 | ATCC 25922 | ATCC 25923 |
| Amoxicillin | <0.5 | 256 | 1 | 256 | 256 | 256 | 1 | 0.25 |
| Ampicillin | 1 | 512 | 512 | 512 | 512 | 512 | 2 | 0.125 |
| Cefotaxime | ≤2 | ≤2 | ≤2 | 512 | 128 | 128 | 0.125 | 2 |
| Ceftiofur | <1 | <1 | 1 | 128 | 64 | 64 | 1 | 1 |
| Erythromycin | 128 | 32 | 16 | 512 | 1024 | 512 | 128 | 0.5 |
| Florfenicol | 8 | 8 | 4 | 64 | 32 | 64 | 8 | 4 |
| Marbofloxacin | 0.062 | 0.25 | 0.25 | 2 | 2 | 4 | 0.25 | 0.25 |
| Norfloxacin | 0.25 | 4 | 0.5 | 4 | 16 | 8 | 0.5 | 0.5 |
| Penicillin G | 8 | 1024 | 32 | >1024 | >1024 | >1024 | 16 | 0.125 |
| Thiamphenicol | 128 | 64 | 128 | 256 | 512 | 512 | 256 | 16 |
| Epicatechin | >1024 | >1024 | >1024 | >1024 | >1024 | >1024 | ≥1024 | ≥1024 |
| Epicatechin gallate | >512 | 512 | >512 | >512 | >512 | >512 | 1024 | ≥1024 |
| Epigallocatechin | 1024 | 512 | 512 | 512 | 512 | 512 | 512 | 32 |
| Gallic acid | 256 | 256 | 256 | 256 | 256 | 256 | 1024 | 128 |
| Hamamelitannin | 512 | 512 | 1024 | 1024 | 1024 | 1024 | 2048 | ≥2048 |

**Table 2:** 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 ½MIC + Gallic acid ½MIC | 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 ½MIC + Hamamelitannin ½MIC | 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 ½MIC + Gallic acid ½MIC | 3.67±1.15[c] | 4.00±1.00[c] |

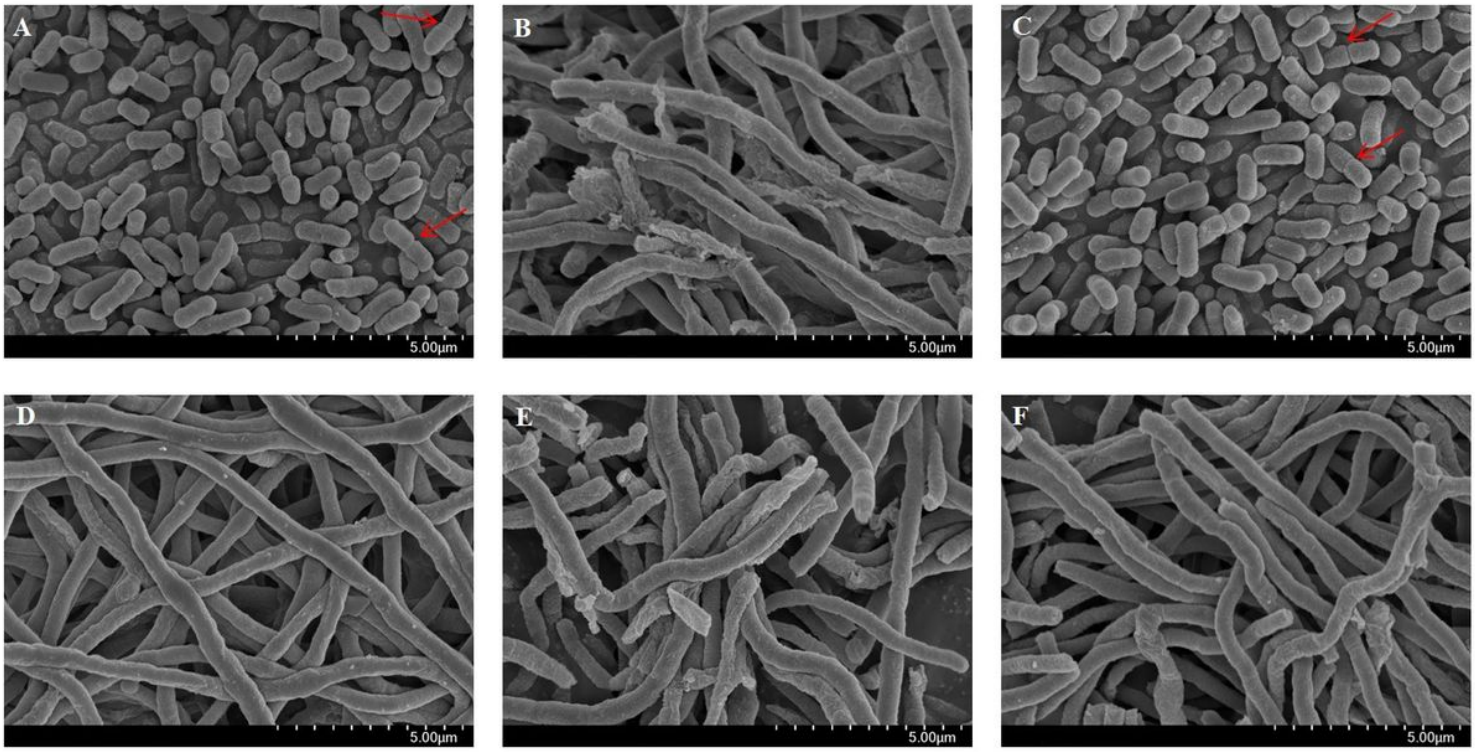
MIC: minimum inhibitory concentration. Different superscript letters indicate statistical significance ($P<0.05$).

## Figures



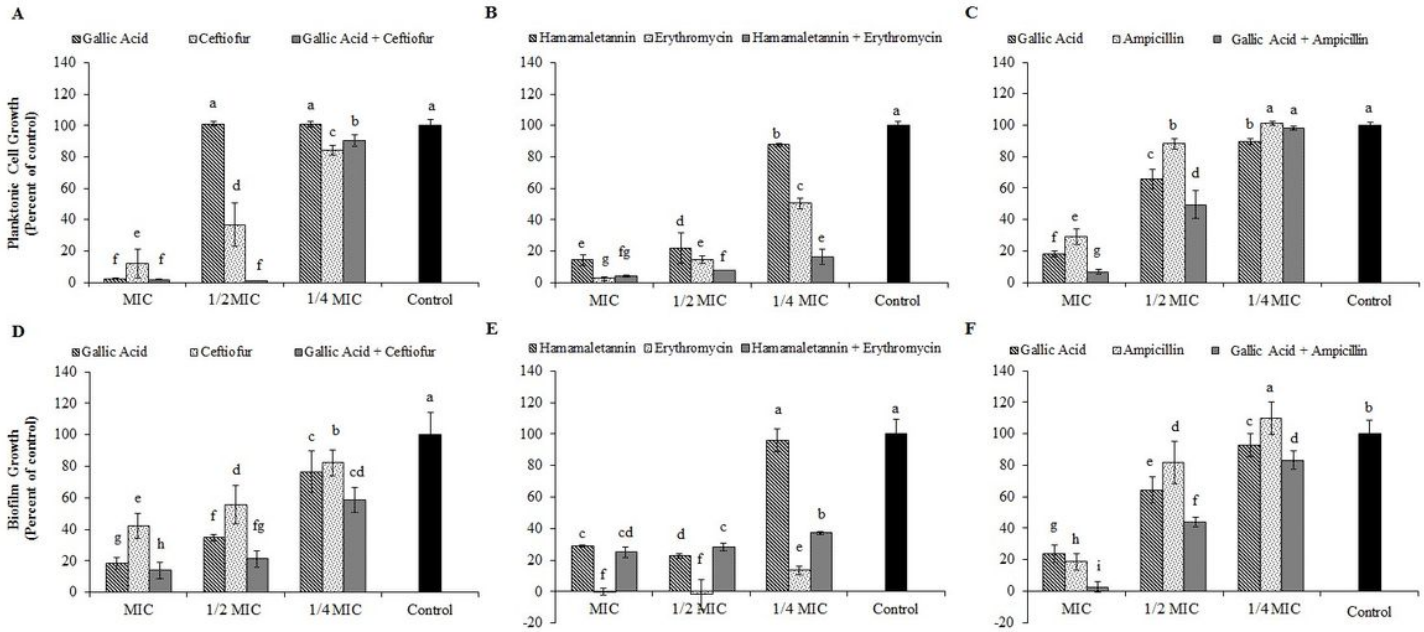

## Figure 1

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 electronic 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. GA, gallic acid; CEF, ceftiofur; 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).

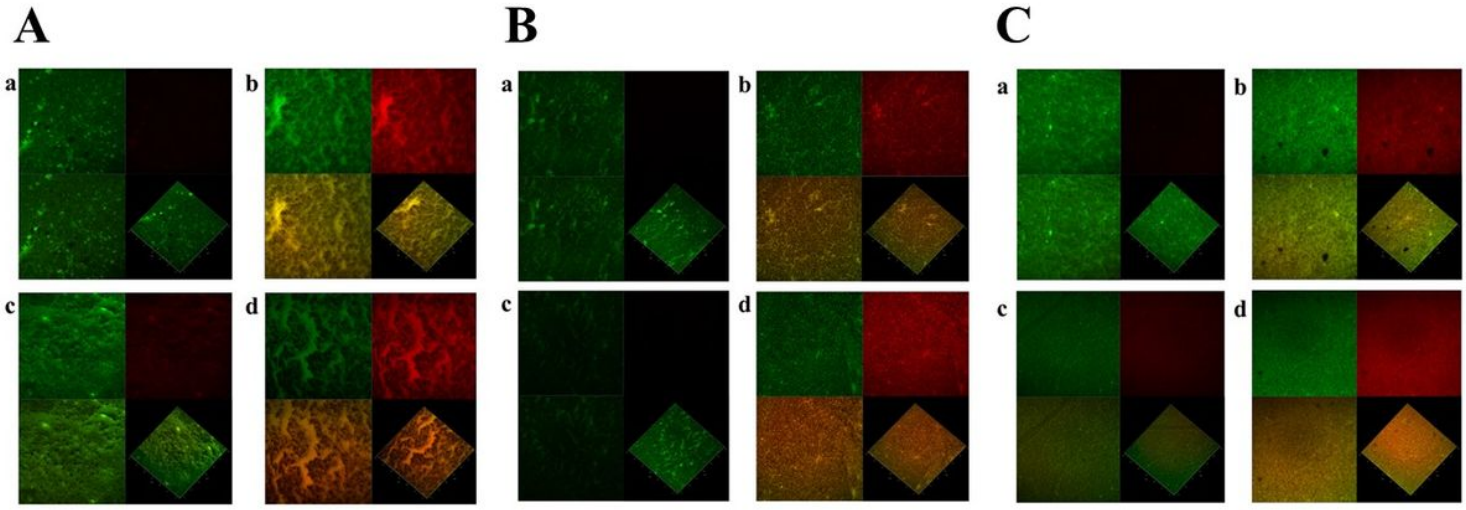



**Figure 4**

Effect of antibacterial combination on the 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 shows two dimensional images.

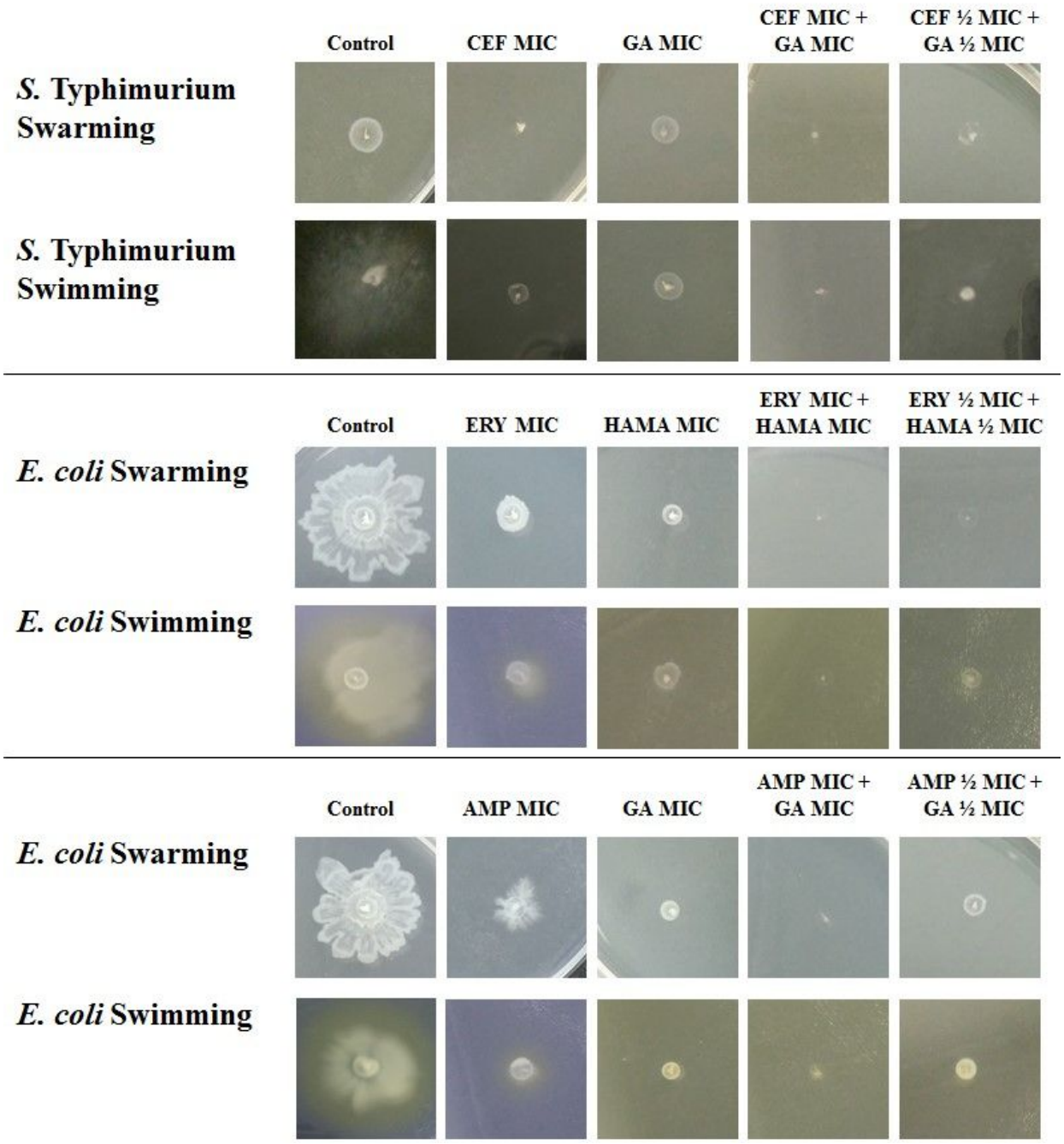



Figure 5

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.

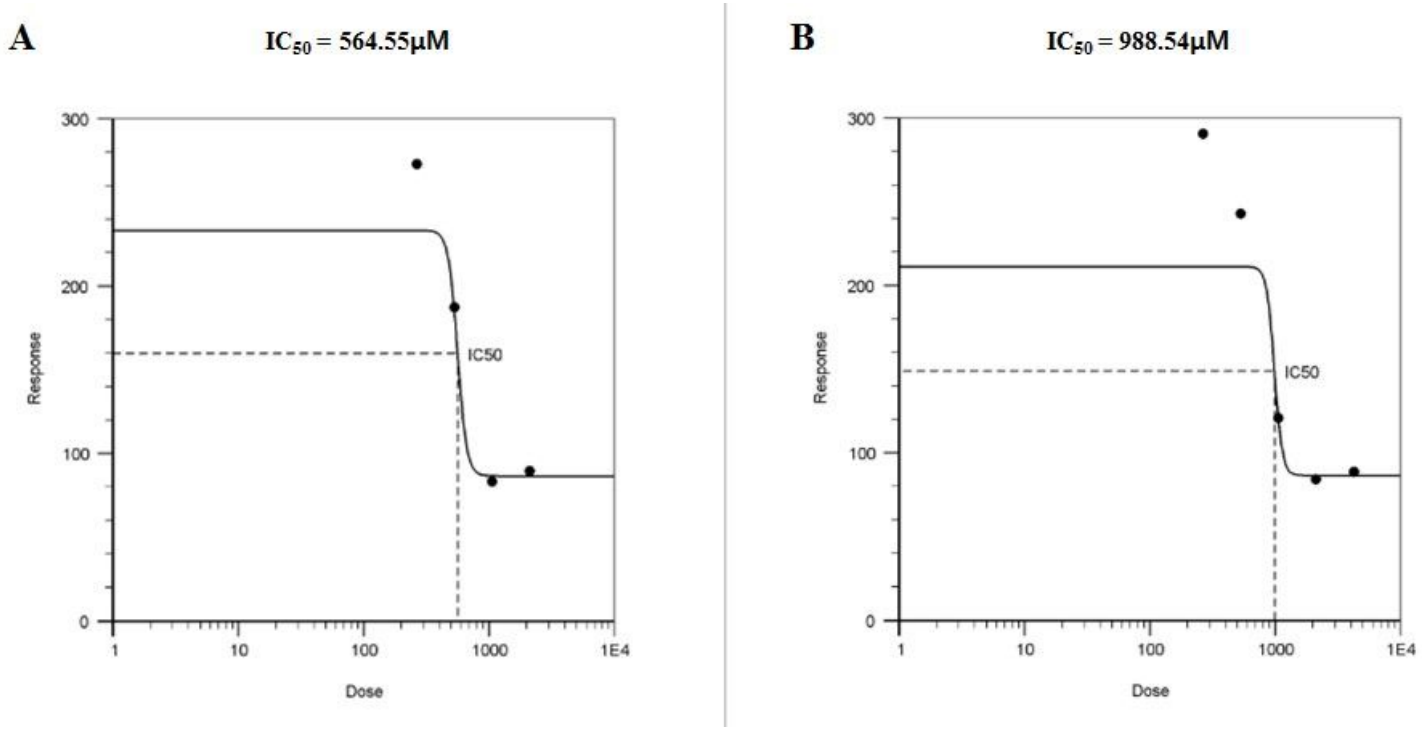



**Figure 6**

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.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.docx
- supplement2.docx