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Original Article

Mutual antimicrobial effect of hibiscus acid and nalidixic acid against multidrug-resistant foodborne bacteria in CD-1 mice

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Abstract

Introduction: The antimicrobial effect of hibiscus acid (HA) alone and in combination with nalidixic acid (NA) on multi-antibiotic-resistant Shiga-like toxin-producing *Escherichia coli* (STEC) and *Salmonella Typhimurium* (ST) was evaluated in CD-1 mice.
Methodology: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for NA and HA were determined against both STEC and ST. Fifteen sets of 6 mice each were utilized: 6 groups were exposed orally to 5 $\log_{10}$ colony forming units of a pool of 3 ST strains, another 6 were exposed to a pool of STEC; and 3 acted as controls. Six hours post-inoculation, specific mice groups received either oral solutions containing HA (2 and 7 mg/mL), or NA (20 and 250 µg/mL), or HA/NA (2 mg/mL HA and 20 µg/mL NA), or isotonic saline. All mice were euthanized on day 5 post infection, and tissues were collected to analyze the numbers of bacteria.
Results: The study determined the MIC and MBC of 7 mg/mL HA; 150 and 250 µg/mL of NA; and two concentrations of HA/NA (1 mg/mL/5 µg/mL and 2 mg/mL/20 µg/mL). Mice that were infected and treated with HA at 7 mg/mL or with HA/NA (2 mg/mL/20 µg/mL) did not have STEC or ST in their fecal samples or in the tissues. However, the pathogens were present in the stool and tissues of infected and untreated mice, and those infected and exclusively treated with NA250, NA20, or HA2 mg/mL.
Conclusions: HA is an alternative for the treatment against antibiotic-resistant pathogenic bacteria.

Key words: antimicrobial; Shiga-toxin; *Salmonella*; synergistic effect.

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Introduction

The rise of antibiotic-resistant pathogenic bacteria has induced a global crisis, undermining the effectiveness of conventional antibacterial drugs, and at times rendering them ineffective [1]. This urgent situation demands innovative strategies to confront bacterial infections. In this context, the diverse plant species' rich biodiversity has seized researchers' attention as a potential reservoir of natural antibacterial agents [2]. These agents hold the potential to yield groundbreaking compounds capable of exerting substantial control over global infections. The therapeutic potential of plants resides within their intricate assortment of secondary metabolites, encompassing alkaloids, flavonoids, terpenoids, and phenolic compounds [2].

These biologically active molecules are synthesized to serve various plant physiological functions and have also showcased their ability to interact with human and microbial systems. Phytochemicals, including flavonoids and polyphenols, have been singled out for their significant impact on human health due to their antioxidant, anti-inflammatory, and antimicrobial properties [3]. Importantly, phytochemicals display substantial bioactivity beyond their effects on human health. Studies, such as those by Portillo-Torres *et al.*, have underscored the varied biological effects of phytochemicals across living organisms, encompassing bacteria, fungi, and even specific animal models [4]. This inter-kingdom bioactivity strongly implies that phytochemicals harbor the potential to counter pathogenic organisms, potentially alleviating the challenges posed by antibiotic resistance.

Shiga-toxin-producing *Escherichia coli* (STEC) and *Salmonella* have arisen as noteworthy health threats, particularly within the United States, casting a shadow on global public health. These two microorganisms, classified as foodborne pathogens, are substantial hazards. The surge in outbreaks attributed to these culprits has sparked significant attention, propelling intensified research and vigilant surveillance efforts to abate their repercussions [5,6].

STEC is distinguished by its capability to incite hemorrhagic colitis, a condition characterized by intense abdominal pain and bloody diarrhea. This bacterium evokes grave concerns due to its propensity for outbreaks, often linked to the consumption of inadequately cooked ground beef, unpasteurized dairy products, and contaminated produce. The virulence of STEC hinges on its secretion of Shiga toxins, culminating in hemolytic-uremic syndrome (HUS), marked by kidney failure and potential enduring health implications. The urgency of comprehending and efficiently managing STEC outbreaks is underscored by outbreak data, as highlighted by Beutin and Martin [7].

Salmonella, another notorious foodborne pathogen, encompasses a diverse array of bacteria capable of inducing gastroenteritis in humans. *Salmonella* infections can range from mild gastrointestinal discomfort to severe dehydration, particularly impacting vulnerable groups like the elderly, young children, and immunocompromised individuals. The roots of *Salmonella* contamination are multifaceted, encompassing poultry, eggs, and even fresh produce. The gravity of the situation was recognized by the Centers for Disease Control and Prevention (CDC) in 2013 [8], accentuating the necessity for comprehensive surveillance and intervention measures to curtail the impact of *Salmonella* outbreaks.

Moreover, an escalating apprehension in the domain of foodborne pathogens is the rise of antibiotic-resistant strains of STEC and *Salmonella*. This disconcerting evolution has been meticulously documented, as evidenced by research undertaken by Fruth *et al.* [9] and Medalla *et al*. [10]. The emergence of antibiotic resistance introduces a convoluted dimension to the existing challenges posed by these pathogens, magnifying potential repercussions on public health and diminishing the efficacy of treatment strategies.

Hibiscus sabdariffa, also known as roselle or sorrel, is a notable subtropical botanical species cultivated across diverse nations, including Mexico, Sudan, India, and Thailand. It is valued for its multifarious applications in traditional ethnomedicine, gastronomy, and beverage production; and this plant has garnered considerable attention owing to its prospective health-enhancing attributes. Recent scientific investigations have identified intriguing facets of *H. sabdariffa*, thereby illuminating its potential in antimicrobial domains. A seminal revelation, expounded in the exploratory endeavor undertaken by Portillo-Torres *et al.* has underscored the pivotal role of hibiscus acid, sourced from the acetonic extraction of *H. sabdariffa* calyces, in endowing the plant with antibacterial efficacy [4]. The momentous breakthrough reported by Portillo-Torres *et al*. has cast light upon the latent therapeutic facets of hibiscus acid [4]. This molecular entity, conjoined with other bioactive constituents inherent to the plant, manifests antibacterial potency, emblematic of a noteworthy stride in augmenting our comprehension of the plant's health-associated attributes. The ambit of antimicrobial attributes is of pertinence considering the escalating global apprehensions associated with antibiotic-resistant pathogens.

Furthermore, leveraging the momentum accrued by research focused on *H. sabdariffa* and its bioactive components, a crucial advancement has been engineered to demystify the safety profile of hibiscus acid. The meticulous study undertaken by Baena-Santillán *et al.* has robustly substantiated the non-toxic character of hibiscus acid [11]. Presently, a lacuna exists in our knowledge concerning the plausible antibacterial impact of hibiscus acid when administered within an animal model inflicted by pathogenic, antibiotic-resistant bacteria. The purview of the present study encompasses the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for nalidixic acid, hibiscus acid, and their composite blend against multidrug-resistant STEC and *Salmonella*. Additionally, the study delves into the evaluation of the antibacterial effect of hibiscus acid when administered in isolation or in conjunction with the antibiotic nalidixic acid to mice afflicted with multidrug-resistant STEC and *S. Typhimurium* infections.

Methodology

Isolation of hibiscus acid

A 1 kg batch of dehydrated calyces of *H. sabdariffa* cultivated in the state of Guerrero, Mexico, was used to obtain hibiscus acid from an acetonic extract as described by Portillo-Torres *et al*. [4].

Bacterial strains

Four multidrug-resistant bacterial strains were isolated from food as follows: *S. Typhimurium* Z2 (resistant to 8 antibiotics), and Z3 (resistant to 6 antibiotics) from carrot [12]; and STEC C4 (Stx2 and resistant to 9 antibiotics) and PM16 (Stx2 and resistant to 10 antibiotics) from raw nopalitos [13] and fresh cheeses made with unpasteurized milk [14], respectively. All bacteria were determined to be resistant to the same 5 antibiotics (amoxicillin–clavulanic acid, amikacin, colistin, erythromycin, and nalidixic acid) according to the protocol indicated by CLSI [15]. It should be noted that according to the CLSI, *Salmonella* and STEC strains are considered resistant to nalidixic acid when the MIC is ≥ 32 µg/mL [15]. It is also important to mention that mutants resistant to rifampicin were used for all studies, and they were obtained from the 4 multidrug-resistant STEC and *Salmonella* described above. Rifampicin-resistant mutant strains (+, Sigma-Aldrich, St. Louis, USA) of *S. Typhimurium* and STEC that were multidrug-resistant to other antibiotics were obtained according to previous reports [16].

Inocula preparation

All 4 of the multidrug-resistant STEC+ and *S. Typhimurium*+ strains were inoculated in trypticase soy broth (TSB) and incubated at 35 °C for 18 hours. The cultures were washed twice in sterile isotonic saline solution (ISS; 0.85% NaCl) by centrifuging at 2000 g for 20 minutes, and the pellets were resuspended in sterile peptone water at about 9 $\log_{10}$ colony forming unites (CFU)/mL. An inocula cocktail of each multidrug-resistant strain was prepared by mixing 1 mL of each washed suspension.

MIC and MBC

To determine the MIC and MBC, we used the macrodilution method using the inocula cocktail of *S. Typhimurium*+ or STEC+ at 1 × 10^5 CFU/mL, as described by Portillo-Torres *et al*. [17]. We applied some variations, such as using trypticasein soy broth (TSB) tubes containing hibiscus acid, nalidixic acid, and hibiscus acid/nalidixic acid mixtures in different concentrations. All treatments were performed in 4 replicates [17].

Fractional inhibitory concentration index (FICI)

To estimate the fractional inhibitory concentration index (FICI) value between hibiscus acid and nalidixic acid, the MIC values obtained with each of the compounds on both pathogenic bacteria were used, in the following equation [18]:

$$\text{FICI} = \frac{\text{MIC nalidixic acid in combination}}{\text{MIC nalidixic acid alone}} + \frac{\text{MIC hibiscus acid in combination}}{\text{MIC hibiscus acid alone}}$$

The FICI results were interpreted as follows: < 0.5 indicated synergism; 0.5–1 indicated an additive effect; >1–2 indicated indifference or no effect, and > 2 indicated antagonism.

Antimicrobial effect in CD-1 mice infected with STEC or S. Typhimurium

The investigation of the antimicrobial effect of hibiscus acid, nalidixic acid, and the hibiscus acid/nalidixic acid mixture, in CD-1 mice infected with rifampicin-resistant mutant strains (+) of STEC or *S. Typhimurium* strains that were multidrug-resistant to other antibiotics was carried out as described previously [17]. Briefly, an inocula cocktail (1 × 10^5 CFU/mL) of STEC+ or *S. Typhimurium*+ was used. Ninety healthy male mice of the CD-1 strain that were 8 weeks of age were used. The experimental protocol involving mice was analysed and approved by the University (UAEH) Ethics Committee for the Care and Use of Laboratory Animals. The MBCs against both STEC+ and *S. Typhimurium*+ were used for inoculation into mice. The concentrations of test solutions used were therefore 2 and 7 mg/mL, 20 and 250 µg/mL, and 2 mg/mL/20 µg/mL for hibiscus acid, nalidixic acid, and hibiscus acid/nalidixic acid, respectively. The 90 mice were divided into 15 groups of 6 mice each (groups I to XV). All groups were maintained for 1 week of adaptation by providing them with standard food and water ad libitum. After this adaptation time, the mice were orally inoculated with STEC+ and *S. Typhimurium*+. The mice were held firmly by the scruff of the neck in a vertical position and inoculated with the R+ pathogen in suspension, antibacterial solution, or saline solution; using an oesophageal cannula attached to a sterile needleless syringe. Mouse Group I was not infected with the pathogenic strains, and no treatment was administered orally (blank, only ISS). Groups II and III were not infected with the pathogenic strains, but they were administered nalidixic acid and hibiscus acid, respectively (uninfected and treated controls). Groups IV, VI, VIII, X, XII and XIV were inoculated orally (0.1 mL) with approximately 1 × 10^4 CFU of *S. Typhimurium*+. Groups V, VII, IX, XI, XIII and XV were inoculated orally (0.1 mL) with approximately 1 ×

10^4 CFU of STEC+. Then, 6 hours after infection, groups IV (*S. Typhimurium*+) and V (STEC+); VI (*S. Typhimurium*+) and VII (STEC+); VIII (*S. Typhimurium*+) and IX (STEC+); X (*S. Typhimurium*+) and XI (STEC+), XII (*S. Typhimurium*+) and XIII (STEC+); and XIV (*S. Typhimurium*+) and XV (STEC+), were orally administered 0.5 mL of ISS (ST + ISS, and STEC + ISS, respectively; positive controls), nalidixic acid 250 µg/mL (ST + NA250, and STEC + NA250, respectively); nalidixic acid 20 µg/mL (ST + NA20, and STEC + NA250, respectively); hibiscus acid 2 mg/mL (ST + HA2 ,and STEC + HA2, respectively); hibiscus acid 7 mg/mL (ST + HA7, and STEC + HA7, respectively), and hibiscus acid/nalidixic acid 2 mg/mL/20 µg/mL (ST + HA/NA 2 mg/mL/20 µg/mL, and STEC + HA/NA 2 mg/mL/20 µg/mL, respectively) solutions. Each of the treatments with the test solutions and the ISS were administered to the mice every 12 hours for 5 days. The STEC+ and *S. Typhimurium*+ in mouse feces were enumerated, and the pathological manifestations were examined exactly as reported previously [17]. Finally, on day 5 of experimentation, all mice were euthanized by cervical dislocation, and the large and small intestine, spleen, and liver were removed, and the presence of STEC+ and *S. Typhimurium*+ were quantified.

Mouse pathological manifestations

Daily observations were conducted to monitor the animals for any physiological and pathological irregularities, including but not limited to weight loss, reduced appetite, signs of weakness or sluggish movement, and mortality, throughout the experimental period.

Bacterial count from mouse feces

The presence of STEC+ and *S. Typhimurium*+ in mouse feces was quantified following the procedure described by Lee *et al*. [19], and Itelima and Agina [20]. Briefly, fecal samples were collected in aseptic conditions from individual cage beds every 8 hours and stored under refrigeration. Specifically, feces from the test animals were directly obtained from the sawdust at the base of each mouse group's cage using sterilized forceps. The collected feces were transferred into plastic bags with hermetic closures. These sealed bags containing fecal specimens from each rodent group were refrigerated at 24-hour intervals after transport to the laboratory. Bacterial counts were quantified for each of the 15 distinct study groups. The sawdust within each of the 9 cages underwent daily replacement and sterilization throughout the stool sample collection period to prevent cross-contamination. The enumeration of pathogenic R+ bacteria was conducted using the pour plate technique on tryptic soy agar (TSA) supplemented with rifampicin (100 mg/L), followed by incubation at 35 ± 2 °C. Colonies from these plates were sub-cultured onto eosin and methylene blue (EMB) agar or brilliant green agar (BGA) to validate the presence of the R+ mutant strain in the TSA-rifampicin plates. These agar media were both supplemented with rifampicin (100 mg/L) and were selective for STEC R+ or *S. Typhimurium* R+, respectively.

Bacterial count from intestines, liver, and spleen of mice

The intestines, liver, and spleen of each mouse were extracted under aseptic conditions. Each organ was placed in a sterile Petri dish. The intestines were then washed with buffer saline phosphate to eliminate the intestinal content; and once they were free of intestinal content, the intestines, liver, and spleen were placed in separate sterile bags with 10 mL of peptone diluent. They were then homogenized manually for 1 minute, vigorously massaging each tissue. The enumeration of pathogenic R+ bacteria in each tissue was conducted as described above.

Statistical analysis

The experiments for MIC/MBC were repeated 3 times. An exploratory data analysis was performed to assess the assumptions of equality of variances and normal distribution of errors of the results obtained from the in vitro antimicrobial activity of hibiscus acid and nalidixic acid. The data was were analyzed using the Statgraphics Centurion XVI statistical program (StatPoint Technologies, Warrenton, VA, USA, 2009) for the one-way analysis of variance. Comparisons of means were performed with the Tukey test, for each experimental section, and a p value of < 0.05 was considered significant.

The results of the analysis of the mice feces were expressed in $\log_{10}$ of CFU of three repetitions for each group per gram. The results of the quantification of the presence of pathogens in the tissues of each individual mouse were expressed with the median and the standard deviation for each group of mice that were subjected to different treatments. These results were analyzed using one-way analysis of variance (ANOVA) with the Statgraphics Centurion XVI statistical program (StatPoint Technologies, Warrenton, VA, USA, 2009). Comparisons of means with the Tukey test were performed for each experimental section, with a significance level of $p < 0.05$ (*) to $p < 0.001$ (***).

Table 1. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and fractional inhibitory concentration index (FICI) values of hibiscus acid, nalidixic acid, and hibiscus acid/nalidixic acid (HA/NA).

Bacteria	Treatments								FICI	Interpretation
	Hibiscus acid			Nalidixic acid			HA/NA			
	MIC (mg /mL)	MBC (mg/mL)	MBC/MIC (mg /mL)	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC (µg/mL)	MIC (mg/mL / µg/mL)	MBC (mg/mL / µg/mL)		
S. Typhimurium	*7 ± 0.0	7 ± 0.0	1	150 ± 0.0	250 ± 0.0	1.6	1 ± 0.0/5 ± 0.0	2 ± 0.0/20 ± 0.0	0.146	Synergism
STEC	7 ± 0.0	7 ± 0.0	1	150 ± 0.0	250 ± 0.0	1.6	1 ± 0.0/5 ± 0.0	2 ± 0.0/20 ± 0.0	0.146	Synergism

* Quadruple average; ± SD; STEC: Shiga-like toxin-producing *Escherichia coli*.

Results

MIC and MBC

The values obtained for the MIC and MBC of hibiscus acid, nalidixic acid, and hibiscus acid/nalidixic acid against *S. Typhimurium*+ and STEC+ are summarized in Table 1. It should be noted that the MIC values that were obtained for hibiscus acid were 7 mg/mL for both *S. Typhimurium*+ and STEC+; while for nalidixic acid, the MIC was 150 µg/mL for both pathogenic strains. However, when the mixture of both agents was tested, the MICs of hibiscus acid/nalidixic acid for STEC+ and *S. Typhimurium*+ were 1 mg/mL and 5 µg/mL, respectively. A similar reduction was observed in the MBC values of hibiscus acid and nalidixic acid, both alone and in mixture (Table 1).

FICI

The FICI determines whether the interaction between hibiscus acid and nalidixic acid in the mixture increases (synergistic or additive effect), decreases (antagonistic effect), or has no effect on the antibacterial activity. FICI values less than 0.5 indicate a synergistic effect, while values of 0.5–1 indicate an additive effect due to the interaction of the compounds in the mixture. The mixture of hibiscus acid with nalidixic acid had a FICI of 0.146 for both *S. Typhimurium* and STEC, thus showing a synergistic effect (Table 1).

Antimicrobial effect in CD-1 mice infected with STEC or S. Typhimurium

The results of the antibacterial activity of hibiscus acid, nalidixic acid, and the hibiscus acid/nalidixic acid mixture in CD-1 mice infected with *S. Typhimurium* R+ or STEC R+ are summarized in Tables 2 and 3. Both *S. Typhimurium*+ and STEC+ were able to colonize mice and replicate in infected mice that were treated with ISS only, nalidixic acid 250 µg/mL, nalidixic acid 20 µg/mL, or hibiscus acid at 2 mg/mL (Table 2). By contrast, when infected mice were administered with hibiscus acid 7 mg/mL or hibiscus acid/nalidixic acid: 2 mg/mL/20 µg/mL, both pathogens were no longer detected in the feces after day 1 (Table 2).

On the other hand, as shown in Table 3, the mice in the groups that were infected with STEC+ or *S. Typhimurium*+ and were administered after infection

Table 2. Effect of treatments in CD-1 mice on the fecal excretion of STEC+ and *S. Typhimurium*+ (CFU/g).

Groups	Treatments	Number of R+ bacteria excreted in the faeces of mice on each day throughout the study (CFU/g)					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
I (Mouse)	ISS	0	0	0	0	0	0
II (Mouse)	Nalidixic acid (250 µg/mL)	0	0	0	0	0	0
III (Mouse)	Hibiscus acid (7 mg/mL)	0	0	0	0	0	0
IV (Mouse + *S. Typhimurium*+)	ISS	0	2×10^3	6×10^3	1×10^4	2×10^4	7×10^5
V (Mouse + STEC+)	ISS	0	3×10^3	7×10^3	8×10^3	4×10^4	6×10^5
VI (Mouse + *S. Typhimurium*+)	Nalidixic acid (250 µg/mL)	0	4×10^3	4×10^3	6×10^3	3×10^2	4×10^2
VII (Mouse + STEC+)	Nalidixic acid (250 µg/mL)	0	2×10^3	5×10^3	8×10^3	4×10^2	6×10^2
VIII (Mouse + *S. Typhimurium*+)	Nalidixic acid (20 µg/mL)	0	2×10^2	1×10^3	8×10^3	1×10^4	8×10^5
IX (Mouse + STEC+)	Nalidixic acid (20 µg/mL)	0	1×10^2	5×10^3	8×10^3	6×10^4	7×10^5
X (Mouse + *S. Typhimurium*+)	Hibiscus acid (2 mg/mL)	0	3×10^2	5×10^3	9×10^3	7×10^4	8×10^5
XI (Mouse + STEC+)	Hibiscus acid (2 mg/mL)	0	1×10^2	4×10^3	7×10^3	1×10^4	6×10^5
XII (Mouse + *S. Typhimurium*+)	Hibiscus acid (7 mg/mL)	0	4×10^2	2×10^1	0	0	0
XIII (Mouse + STEC+)	Hibiscus acid (7 mg/mL)	0	1×10^2	0	0	0	0
XIV (Mouse + S. Typhimurium+)	HA/NA 2 mg/mL/20 µg/mL	0	3×10^2	0	0	0	0
XV (Mouse + STEC+)	HA/NA 2 mg/mL/20 µg/mL	0	2×10^2	0	0	0	0

* Represents the average of the three-repeat count of the same stool sample of each group collected every 24 hours. CFU: colony forming units; ISS: isotonic saline solution; HA/NA: hibiscus acid/nalidixic acid; STEC+: Shiga-toxin-producing *Escherichia coli*+; +: rifampicin-resistant.

Table 3. Clinical signs and mortality observed in groups of mice infected and not infected with *S. Typhimurium*+ and STEC+ during the experiment.

Groups	Treatments	Clinical symptoms or mortality			
		Loss of weight	Loss of appetite	Body weakness/slow movement	Mortality rate
I (Mouse)	ISS	*0/6	0/6	0/6	0/6
II (Mouse)	Nalidixic acid (250 µg/mL)	0/6	0/6	0/6	0/6
III (Mouse)	Hibiscus acid (7 mg/mL)	0/6	0/6	0/6	0/6
IV (Mouse + *S. Typhimurium*+)	ISS	6/6	6/6	6/6	0/6
V (Mouse + STEC+)	ISS	6/6	6/6	6/6	0/6
VI (Mouse + *S. Typhimurium*+)	Nalidixic acid (250 µg/mL)	6/6	6/6	6/6	0/6
VII (Mouse + STEC+)	Nalidixic acid (250 µg/mL)	6/6	6/6	6/6	0/6
VIII (Mouse + *S. Typhimurium*+)	Nalidixic acid (20 µg/mL)	6/6	6/6	6/6	0/6
IX (Mouse + STEC+)	Nalidixic acid (20 µg/mL)	6/6	6/6	6/6	0/6
X (Mouse + *S. Typhimurium*+)	Hibiscus acid (2 mg/mL)	6/6	6/6	6/6	0/6
XI (Mouse + STEC+)	Hibiscus acid (2 mg/mL)	6/6	6/6	6/6	0/6
XII (Mouse + *S. Typhimurium*+)	Hibiscus acid (7 mg/mL)	1/6	1/6	1/6	0/6
XIII (Mouse + STEC+)	Hibiscus acid (7 mg/mL)	2/6	2/6	2/6	0/6
XIV (Mouse + *Typhimurium*+)	HA/NA 2 mg/mL/20 µg/mL	1/6	1/6	1/6	0/6
XV (Mouse + STEC+)	HA/NA 2 mg/mL/20 µg/mL	2/6	2/6	2/6	0/6

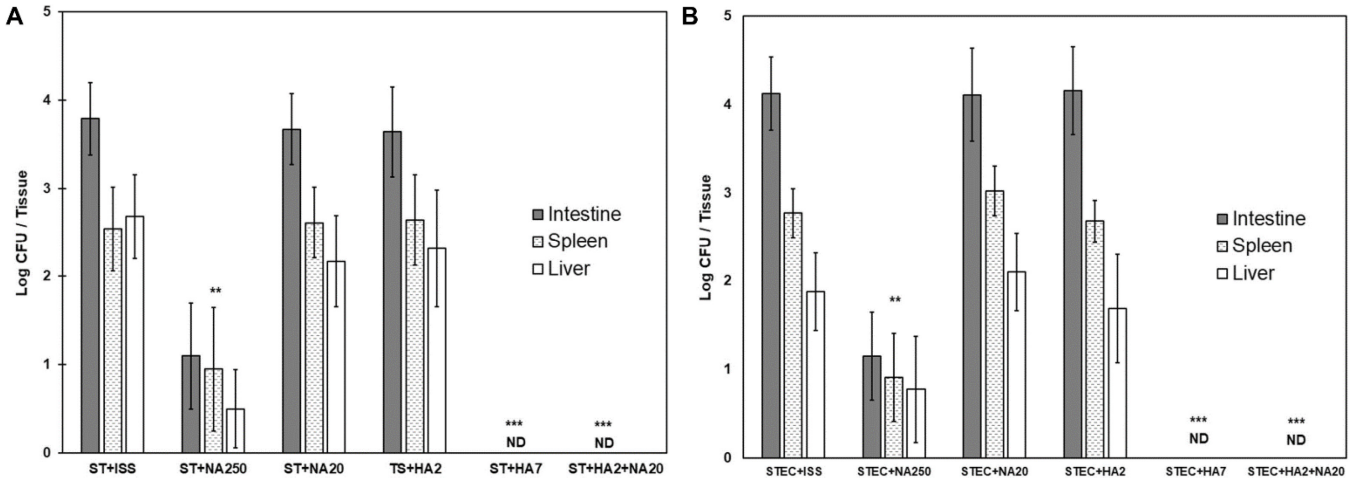
ISS: isotonic saline solution; HA/NA: Hibiscus acid/nalidixic acid; STEC+: Shiga-toxin-producing *Escherichia coli*+; +: rifampicin-resistant; * Number of affected mice/total number of mice in each group.

with ISS-only, nalidixic acid 250 µg/mL, nalidixic acid 20 µg/mL, or hibiscus acid 2 mg/mL, developed clinical signs during the study. In contrast, in the case of mice that were infected with STEC+ or *S. Typhimurium*+ but were administered with hibiscus acid 7 mg/mL or hibiscus acid/nalidixic acid mixture (2 mg/mL/20 µg/mL), only 2 mice from each group exhibited any symptoms in the first 2 days following infection; but gradually recovered after administration of hibiscus acid or hibiscus acid/nalidixic acid mixture.

Bacterial count from intestines, liver, and spleen of mice

Figure 1 shows the results of the presence of both *S. Typhimurium* and STEC in all the 3 types of mice tissues that were examined. In general, it was observed that the 2 pathogens were found in the tissues of infected mice that were treated with ISS-only, nalidixic acid 250 µg/mL, nalidixic acid 20 µg/mL, or hibiscus acid 2 mg/mL. The pathogens were found in the tissues of mice from the groups: ST + ISS (positive control, which was infected and only treated with ISS), ST +

Figure 1 A. Number of CFU of *S. Typhimurium* in different tissues obtained from infected mice that were subjected to different treatments during the study and subsequently euthanized; **B.** Number of CFU of STEC in different tissues obtained from infected mice that were subjected to different treatments during the study and subsequently euthanized.



ST + ISS, mice infected and administered with isotonic saline solution; ST + NA250, mice infected and administered with 250 µg/mL nalidixic acid; ST + NA20, mice infected and administered with 20 µg/mL nalidixic acid; ST + HA2, mice infected and administered with 2 mg/mL hibiscus acid; ST + HA7, mice infected and administered with 7 mg/mL of hibiscus acid; ST + HA2 + NA20, mice infected and administered with a mixture of 2 mg/mL of hibiscus acid plus 20 µg/mL of nalidixic acid. The bars represent geometric means ± SEM of bacteria recovered from each tissue (CFU/tissue). **$p < 0.01$ compared between treatments ST + HA250 with respect to ST + ISS, ST + NA20, and ST + HA2; ***$p < 0.001$ compared between treatments ST + HA7 or ST + HA2 + NA20 with respect to ST + ISS, ST + NA250, ST + NA20, and ST + HA2. ND: The pathogen was not detected. CFU: colony forming units; HA: hibiscus acid; ISS: isotonic solution; NA: nalidixic acid; SEM: standard error of mean; ST: *Salmonella Typhimurium*.

NA250 (group infected and treated with nalidixic acid at a concentration of 250 µL/mL), ST + NA20 (group infected and treated with nalidixic acid at a concentration of 20 µL/mL), and ST-HA2 (group infected and treated with hibiscus acid at a concentration of 2 mg/mL). In contrast, none of the pathogens were detected in the tissues of the ST + HA7 group (infected group and treated with hibiscus acid at a concentration of 7 mg/mL) and ST-HA2 + NA20 (infected group and treated with a mixture of hibiscus acid at a concentration of 2 mg/mL and nalidixic acid at a concentration of 20 µL/mL) (Figure 1). Pathogens were also not found in the tissues of the negative control groups (mice that were not infected with the pathogens).

The highest concentration of both pathogens was detected in the intestines (around 4 $\log_{10}$) of all the groups of rodents where both pathogens were detected. A significantly lower concentration of both pathogens was quantified in the spleen than in the intestine (around 3 $\log_{10}$). Different concentrations of pathogens were observed in the liver: approximately 3 $\log_{10}$ for *S. Typhimurium* and 2 $\log_{10}$ for STEC (Figure 1).

Discussion

MIC and MBC

In this investigation, the antimicrobial susceptibility profiles of multidrug-resistant STEC and *S. Typhimurium* strains, have demonstrated markedly elevated MIC and MBC levels when contrasted with those exhibited by their antibiotic-sensitive strains [15]. These results reaffirm that the *Salmonella* and STEC strains used were resistant to nalidixic acid [15]. This phenomenon implies that an elevated quantity of nalidixic acid would be imperative to effectively combat infections engendered by these virulent strains, thereby engendering an inherent jeopardy attributed to its intrinsic cytotoxicity. It is well-substantiated within the literature that even at dosages conventionally administered for controlling infections mediated by nalidixic acid-sensitive bacterial strains, an appreciable degree of toxicity is inherently associated with nalidixic acid [21].

To reduce the hazards presented by certain antibiotics — exemplified by nalidixic acid — to human and animal health and to amplify their efficacy against multidrug-resistant bacterial adversaries, we proffer an innovative strategy encompassing the synergistic amalgamation of antibiotics, inclusive of nalidixic acid, with hibiscus acid. In the scope of our investigation, the coalescence of hibiscus acid with nalidixic acid has yielded meritorious revelations. Conspicuously, the MIC and MBC metrics pertinent to the amalgamated formulation against both multidrug-resistant STEC and *S. Typhimurium* strains have evinced a noteworthy and remarkable reduction in contrast to the MIC and MBC values attained through the administration of each discrete compound.

These results underscore the potential of hibiscus acid-antibiotic combinations as promising agents against multidrug-resistant bacteria. Furthermore, such combinations hold the prospect of reducing toxicity through the lowering of antibiotic concentrations.

Based on the results that we previously obtained [4], it is very possible that hibiscus acid, by affecting the permeability of the membrane, favors the entry of nalidixic acid into the bacterial cell and thus may inhibit DNA synthesis [22]. This hypothesis finds resonance within the broader realm of research exploring the synergistic interplay between plant-derived compounds and conventional antibiotics. Notably, several studies have probed similar phenomena, elucidating how plant compounds can modify bacterial membrane properties, thereby potentiating the cellular uptake of antibiotics [23]. These alterations in membrane permeability, in turn, can heighten the effectiveness of antibiotics in inhibiting bacterial growth [23]. The hypothesis posited in our study, suggesting that hibiscus acid's influence on membrane permeability promotes the entry of nalidixic acid into bacterial cells to potentially inhibit DNA synthesis, finds alignment with analogous studies exploring the synergy between plant-derived compounds and antibiotics.

The growing body of evidence underscores the promise of leveraging plant-derived compounds to enhance the efficacy of conventional antibiotics in combating multidrug-resistant bacterial infections.

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Synergistic and additive interactions between two antibacterial components have been reported to improve antibacterial efficacy compared to when used alone [24]. However, mixtures of antibiotics and plant extracts with antibacterial properties do not always result in an additive or synergistic effect. For example, Farooqui *et al.* reported that the mixture of chloramphenicol with the methanolic extract of *Camelia sinensis* did not present an antibacterial effect on *S. Typhimurium* that was greater than that of each one separately [25].

Drug or antibiotic mixture therapy with plant derivatives is used to expand the antibacterial spectrum, reduce toxicity, and decrease antibacterial resistance during treatment [26]. Plant compounds have gained

attention for their potential to augment the effects of antibiotics. Studies by Vipin *et al.* explored the synergistic potential of plant compounds with antibiotics, showcasing how these combinations can lower antibiotic doses while maintaining or even enhancing antimicrobial activity [27]. This approach could be particularly beneficial for nalidixic acid, helping to achieve therapeutic outcomes with reduced toxicity effects. Moreover, plant compounds have been shown to possess protective properties against the toxic effects of antibiotics. Research by Karamova *et al.* demonstrated the protective effects of *Chelidonium majus* L. against the genotoxic effect of nalidixic acid [28].

In our study, the results show the possibility of significantly reducing the concentration of nalidixic acid for the treatment of infections if it is mixed with hibiscus acid (Table 1). It is known that nalidixic acid can be toxic at the concentrations used for the treatment of bacterial infections in humans and animals, and can cause serious conditions [28]. Considering these concerns, the need to reduce nalidixic acid dosage in humans and animals is evident. However, achieving this reduction while maintaining therapeutic efficacy remains a challenge. As we mentioned above, one promising approach is the combination of nalidixic acid with hibiscus acid. This not only aims to enhance the antibiotic's efficacy but also to mitigate its toxic impact.

Antimicrobial effect in CD-1 mice infected with STEC or S. Typhimurium

The present study shows that hibiscus acid at the concentration tested (minimum bactericidal dose) has an antimicrobial effect in animals infected by both pathogens. In addition, the mixture of hibiscus acid with nalidixic acid showed an antimicrobial effect in the mice examined (Table 2). It should be remembered that in this mixture the concentration of hibiscus acid used was lower than when it was tested in pure form. This result suggests that hibiscus acid potentiates the effect of nalidixic acid and that due to this interaction, a very low concentration is needed in a mixture with hibiscus acid to inactivate both pathogens, even when they are resistant to nalidixic acid alone. These results suggest that even though nalidixic acid presents toxicity to humans and animals at the doses in which it is generally administered, it could continue to be used at a lower and non-toxic dose if mixed with plant antimicrobials such as hibiscus acid.

The growing prevalence of antibiotic-resistant bacterial strains underscores the urgent need for innovative approaches to combat these pathogens. The exploration of plant-derived compounds as potential synergistic partners with antibiotics has garnered attention due to their antimicrobial properties and potential to enhance treatment efficacy.

It is worth noting that while the exact mechanisms underlying these synergistic effects are not fully elucidated, the results collectively highlight the potential benefits of combining plant compounds, like hibiscus acid, with antibiotics. This strategy not only offers a way to enhance antimicrobial efficacy but also holds promise in reducing the necessary antibiotic dosages, thereby potentially mitigating the risk of antibiotic resistance. The combination of plant extracts or compounds with antibiotics presents a way for addressing infections caused by antibiotic resistant pathogenic bacteria. The studies by Voukeng *et al.* underscore the potential of such combinations in enhancing treatment efficacy and potentially contributing to the global effort to combat antibiotic resistance [29].

It is important to note that, throughout the course of the study, no adverse effects were observed in CD-1 mice treated with HA, NA, or the HA/NA combination. The absence of adverse events supports the safety profile of HA as an alternative treatment against antibiotic-resistant pathogenic bacteria. However, it is important to note that this study primarily focused on the antimicrobial efficacy of the compounds. Therefore, further studies are recommended to include a more comprehensive evaluation of potential adverse effects, utilizing larger sample sizes and longer treatment periods.

Bacterial count from intestines, liver and spleen of mice

The study outcomes indicate the presence of both *S. Typhimurium* and STEC in all examined tissues of mice across diverse treatment groups, except for those administered solely with hibiscus acid at a concentration of 7 mg/mL (ST + AH7) and the combined treatment group of hibiscus acid at 2 mg/mL and nalidixic acid at 20 µL/mL (ST + HA2 + NA20). This suggests an antimicrobial effect of hibiscus acid at these concentrations, either individually or in combination with nalidixic acid. Furthermore, both STEC and *Salmonell*a were identified in the liver and spleen of mice treated with solutions other than ST-AH7 or ST-AH2 + NA20, underscoring the potential for systemic dissemination and the associated risks of pathogenic translocation. It should be noted that, the concentration of *Salmonella* and STEC in the tissues of infected mice that were treated with 250 µL/mL of

nalidixic acid was significantly lower, although both the pathogens were isolated from the tissues, (Figure 1).

The translocation of STEC and *Salmonella* to the liver and spleen represents a significant concern due to the potential for systemic infection and associated complications. The liver and spleen play crucial roles in immune surveillance and pathogen clearance; however, the presence of these pathogens in these organs suggests the evasion of host defenses and the establishment of systemic infection. Moreover, the liver serves as a vital organ involved in detoxification and metabolic processes, and the infiltration of pathogens can lead to hepatic dysfunction and systemic toxicity. Similarly, the spleen is integral to the immune response, and the presence of pathogens may impair its function, predisposing the host to secondary infections and systemic inflammation.

Several studies have demonstrated the ability of STEC and *Salmonella* to translocate to extraintestinal sites, including the liver and spleen, leading to severe systemic infections and septicemia. For instance, a study by Shu and Gill reported the translocation of STEC to the liver and spleen in a murine model, highlighting the systemic dissemination of these pathogens and the associated morbidity and mortality [30].

Furthermore, the synergistic antimicrobial effect observed with the combination of hibiscus acid and nalidixic acid suggests a potential strategy for enhancing therapeutic efficacy and mitigating the risk of pathogenic infection and translocation. Previous studies have investigated the synergistic interactions between natural compounds and conventional antibiotics, demonstrating enhanced antimicrobial activity against multidrug-resistant pathogens. For example, a study by Wang *et al.* demonstrated the synergistic effect of eugenol and colistin against clinical isolates of colistin-resistant *E. coli*, emphasizing the potential of combination therapy in combating antimicrobial resistance [31].

An alternative approach for future research could involve elucidating the optimal dosing regimen and administration route for the hibiscus acid/nalidixic acid combination in human and animals. This could entail conducting dose-response studies to determine the most effective concentrations of hibiscus acid and nalidixic acid for combating multidrug-resistant pathogens while minimizing potential adverse effects.

Additionally, exploring the pharmacokinetics and pharmacodynamics of the hibiscus acid/nalidixic acid combination in humans and animals would provide valuable insights into its absorption, distribution, metabolism, and excretion properties. This could involve studying the bioavailability and tissue distribution of both compounds individually and in combination, as well as assessing their potential for drug-drug interactions.

Furthermore, conducting field trials or longitudinal studies in humans and animals to evaluate the practicality and effectiveness of administering the hibiscus acid/nalidixic acid combination under real-world conditions would be invaluable.

Conclusions

We assessed the antibacterial efficacy of nalidixic acid, hibiscus acid, and their combination against multidrug-resistant STEC and *S. Typhimurium* using CD-1 mice. Notably, the combination demonstrated reduced MIC and MBC values compared to the individual compounds, indicating a potential synergistic effect. The observed pathogen elimination and increased disease recovery rate in treated mice signify a significant advancement in combating antibiotic-resistant infections. These findings hold promise for innovative treatment strategies, shaping the landscape of antibacterial research. The implications of this study extend to animal and human health, offering potential alternatives to address multidrug-resistant pathogens; thus contributing to the ongoing battle against antibiotic resistance. However, further studies are required.

Furthermore, although in this study no adverse effects were detected in mice treated with hibiscus acid, nalidixic acid, or their combination, future research should consider a more extensive assessment of potential adverse effects, which would contribute to a better understanding of the safety and feasibility of using hibiscus acid in long-term treatments.

To the best of our knowledge, this the first report in the literature on the antimicrobial effect of hibiscus acid, both alone and in a mixture with an antibiotic, in an animal model.

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Conflict of interests
No conflict of interests is declared.

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