

Characterization of antibacterial properties of common spices

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SUMMARY

Antibiotics are used by physicians to treat bacterial infections. However, due to overuse, antibiotic-resistant strains of bacteria now threaten the efficacy of many antibiotic types. We tested whether 11 commonly used spices could inhibit growth of the gram-negative bacteria, *E. coli*. We tested cinnamon, clove, thyme, oregano, cumin, garlic, black pepper, rosemary, basil, and ginger, all of which have previously been suggested to have antibiogenic properties. When these spices were diluted in Luria-Bertani (LB) agar, five spices (clove, cinnamon, garlic, sage, and thyme) inhibited growth completely. After a second round of experimentation using these five spices in liquid LB cultures, clove inhibited growth at a low concentration most effectively and four other spices (cinnamon, garlic, thyme, and sage) also effectively inhibited growth. The results of this study suggest that certain spices and herbs have antibacterial effects that can inhibit growth of *E. coli* and that these spices could show similarly promising activity towards other bacteria.

INTRODUCTION

Bacterial infection is resurging as one of the most dangerous challenges facing the medical establishment. Americans spend about 55 to 70 billion dollars per year on antibiotics, yet these antibiotics are becoming increasingly ineffective as illness-causing bacteria gain resistance to the prescribed drugs (1). This increasing resistance is making it harder for physicians to prescribe antibiotics that can treat bacterial infections. Additionally, some bacterial strains have started to become resistant not only to single antibiotics, but to multiple classes of antibiotics, making these infections very difficult to treat and dangerous to public health (2).

Antibiotic resistance occurs when bacteria evolve to prevent the deleterious effects of antibiotics on their growth and survival. There are two means by which bacteria resist the effects of antibiotics: an innate ability of the bacterial species to resist a given antibiotic (intrinsic resistance) or the acquisition of resistance through either random DNA mutation or horizontal transfer of resistance genes from another bacterial species (acquired resistance) (4). One commonly proposed hypothesis about the origin of innate resistance is that because some bacteria produce antibiotic compounds to compete with other bacterial species, these bacteria should have resistance to that antibiotic, so they do

not kill themselves. These resistance genes may have first been transferred to pathogenic bacteria through horizontal transfer (3). However, resistance has now been propagated through random DNA mutation due to the pressures of the overuse of antibiotics (5).

For centuries before the discovery of antibiotics, humans relied on medicines made from herbs and spices. Furthermore, people used and passed down remedies which typically consisted of substances people ate or added to their food (6). With the rise of antibiotic resistance, there has been an increased interest in plants as an economical and renewable source of antimicrobials with rich chemical diversity (7).

Most bacteria that are resistant to one or more antibiotics are gram-negative, meaning that they have only a thin layer of peptidoglycan between their inner and outer cell membrane. Since most antibiotics have to pass through the outer membrane of bacteria, any change in the outer membrane by the gram-negative bacteria can create antibiotic resistance (4). Therefore, many research studies that test novel antibiotics use gram-negative bacteria, such as *E. coli*, as their model system. Unfortunately, antibiotic resistance is still a major problem for many gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA).

We evaluated whether spices and herbs could be used as an effective method to suppress bacteria growth and in turn antibiotic resistant bacteria. We tested 11 spices and herbs: cinnamon, clove, thyme, oregano, cumin, garlic, black pepper, rosemary, basil, sage, and ginger. We used these 11 spices, because each of them was previously suggested to have antibiogenic properties; as a result, we wondered which ones were most effective at suppressing growth of *E. coli* bacteria (8). While we hypothesized that all the spices would be capable of suppressing bacteria growth in a concentration-dependent manner, we found that only five spices are most effective at preventing bacteria growth in both Luria-Bertani (LB) agar and liquid cultures: clove, cinnamon, garlic, sage, and thyme. This result is a good sign that some spices and herbs could potentially be useful in combating the rise of antibiotic resistant bacteria.

RESULTS

To determine which spices might affect *E. coli* growth, we grinded each of the 11 spices to a fine consistency, mixed them into LB agar at 5% concentration, and made LB plates

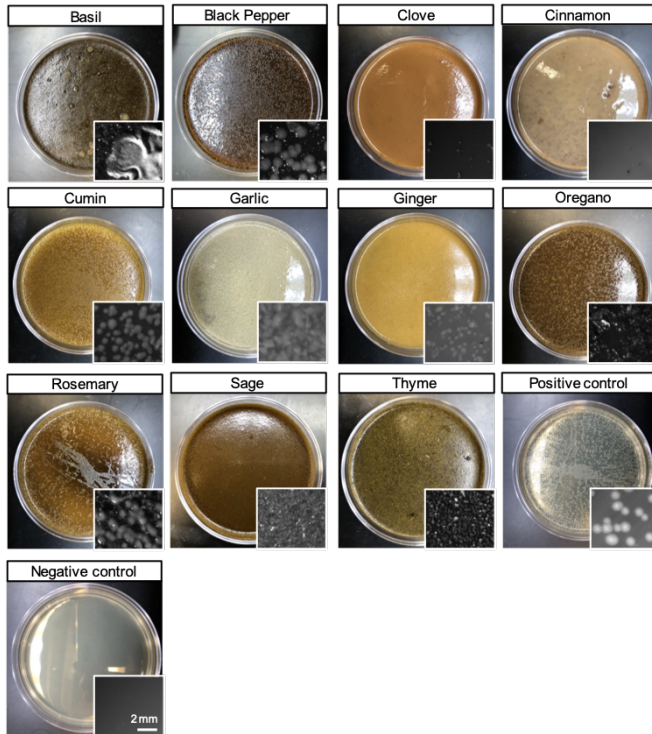


Figure 1: Images of *E. coli* grown with spice and herbs diluted in agar at 5% concentration. The images were acquired 18-24 hours after inoculation, following incubation of agar plates at 37°C.

using this agar. Equal amounts of *E. coli* were spread on each plate, three replicates of which were incubated overnight at 37°C. We chose to use a high, 5% concentration of spice in LB agar to ensure that spices with antibacterial properties would sufficiently inhibit bacterial growth as to be unambiguous. We chose a positive control of *E. coli* bacteria without any spice to ensure that the bacteria would grow normally. As a negative control, we conducted all steps of the experiment without adding *E. coli* to normal LB agar.

We found that four spices did not prevent bacterial growth: pepper, rosemary, cumin, and ginger (**Figure 1**). Interestingly, not all growth looked the same, and growth on the plates varied in both colony density and single colony size. Of these spices, ginger-treated plates had the lowest average colony density at 2.5 ± 0.4 (mean \pm SEM) colonies per square millimeter (C/mm^2), while cumin, pepper, and rosemary exhibited $4.1 \pm 0.2 C/mm^2$, $2.7 \pm 0.7 C/mm^2$, and $3.5 \pm 0.5 C/mm^2$, respectively (**Figure 2**). Plates mixed with 5% basil exhibited few if any *E. coli* colonies, and instead showed white 'bubble' growths that appeared fungal rather than bacterial. These colonies were irregular and not possible to quantify. The positive control plates exhibited a colony density of $2.5 \pm 0.7 C/mm^2$, with a representative colony size of $0.86 mm^2$ (**Figure 3**). Interestingly, agar plates treated with ginger and cumin both produced visually smaller colonies than positive control plates, with a representative colony measured at $0.15 mm^2$ and $0.47 mm^2$, respectively (**Figure 3**).

We found that the only plates that did not show any

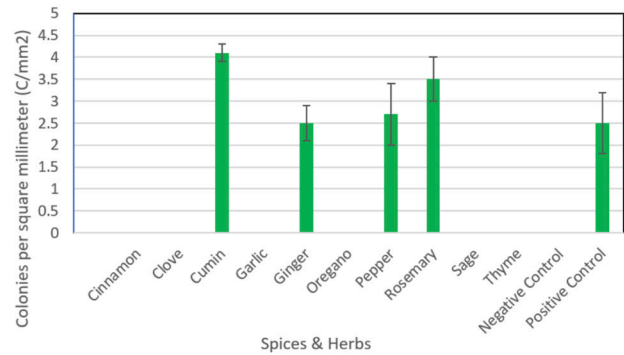


Figure 2: Mean *E. coli* colony density from cultures treated with spice and herb treatments, 18-24 hours after inoculation. Errors bars represent SEM, n=3 plates per treatment.

bacterial growth were clove, cinnamon, garlic, oregano, sage, and thyme, and the negative control. These plates lacked bacterial colonies entirely on all three replicate plates (**Figures 1 & 2**). We concluded that these five spices (cinnamon, garlic, thyme, sage, and clove) demonstrated the most potent antibacterial qualities.

Encouraged by the apparent potency of clove, cinnamon, garlic, oregano, sage, and thyme, we performed a follow-up experiment to determine whether these spices are effective at inhibiting bacterial growth at lower concentrations. The purpose of this second experiment was to find the minimum inhibitory concentration (MIC) of these spices, or the lowest amount of these spices needed to prevent bacteria growth. To find the MIC, we used a spectrophotometer to measure the optical density (OD) of *E. coli* LB cultures with each spice at increasing logarithmic concentrations of 0.01%, 0.1%, and 1%. We mixed the different concentrations of each spice in LB (with three replicates) and grew *E. coli* bacteria in the mixtures (**Figure 4A**). After incubation overnight, we centrifuged the bacteria into a pellet, resuspended it, and measured optical density using a spectrophotometer, which measures how much light of various wavelengths is transmitted through a liquid sample. By measuring light absorbance at 600nm, we estimated how many bacteria cells in colony-forming units (CFU) were present in the liquid, therefore gaining a measure of antibacterial activity for each spice.

We found that cinnamon, clove, sage, thyme, and garlic all inhibited bacteria growth when used at 1% concentration ($p < 0.05$, as assessed by Student's T-test), but not at 0.1% nor 0.01% (**Figure 4B**). Clove was the most effective at suppressing bacterial growth, and at 0.1% concentration also inhibited growth as compared to control ($p=0.012$, as assessed by Student's T-test), whereas garlic, sage, cinnamon, and thyme did not show statistically-significant decreases in OD600 at this concentration. Surprisingly, 1% oregano was ineffective at suppressing bacteria growth, and *E. coli* cultures with oregano exhibited higher bacteria concentrations than the positive control (0.393 vs. 0.283 OD600; $p=0.024$).

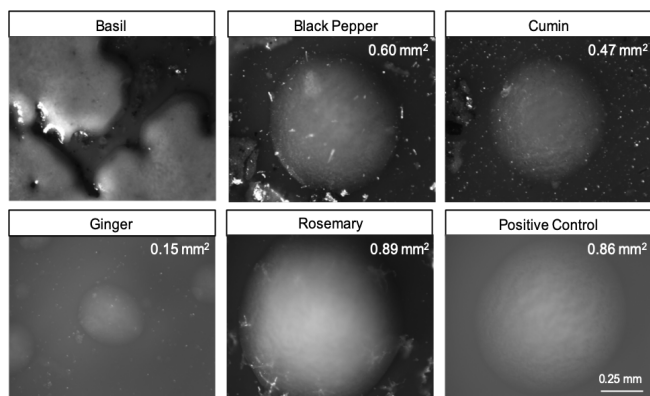


Figure 3: Representative images of individual colonies grown on agar plates with 5% spice treatment. Ginger and cumin treatments resulted in colonies smaller than those of control plates.

(**Figure 4**). The negative control demonstrated little to no bacteria growth, as assessed by visual clarity and OD600 measurements (**Figure 4**).

DISCUSSION

We hypothesized that all spices would be capable of suppressing *E. coli* growth, although in a concentration-dependent manner. Instead, we found that only five spices were effective in suppressing growth, and with the concentrations that we tested, growth suppression occurred in a binary fashion (growth or no growth). However, the fact that some spices and herbs were able to inhibit most bacteria growth at 1% concentrations suggests that other approaches to using spices – such as using fresh spices or extracting the active ingredients – might help in finding solutions to the antibiotic-resistance crisis. Further research that identifies these active ingredients might provide the basis for new antibiotic strategies to be integrated into the medical field. These new antibiotics could even be used in formulations with current antibiotics to overcome antibiotic-resistant bacteria strains.

Metabolites of the spice plants are likely the reason behind their antimicrobial properties (9). Typically, metabolites can inhibit bacterial growth by binding to bacterial proteins or by reducing the pH of the bacteria, which kills them (7). Additionally, metabolites possess antimicrobial mechanisms that can damage microbial membranes, impair cellular metabolism, and lower microbial toxin production (9). Interestingly, plant extracts have antimicrobial activity with potential to help prevent cell wall construction, impede microbial DNA replication, and constrain energy production (9). Modern medicine could harness these metabolites to prevent antibacterial resistance (9).

Previous research efforts have focused on combating the rise of antibiotic resistance or finding an alternative to antibiotics altogether. For example, researchers have studied the use of bacteriophages to combat the antibiotic-resistant bacteria, which can target and kill a specific bacteria strain while not harming eukaryotic cells of the host animal (4).

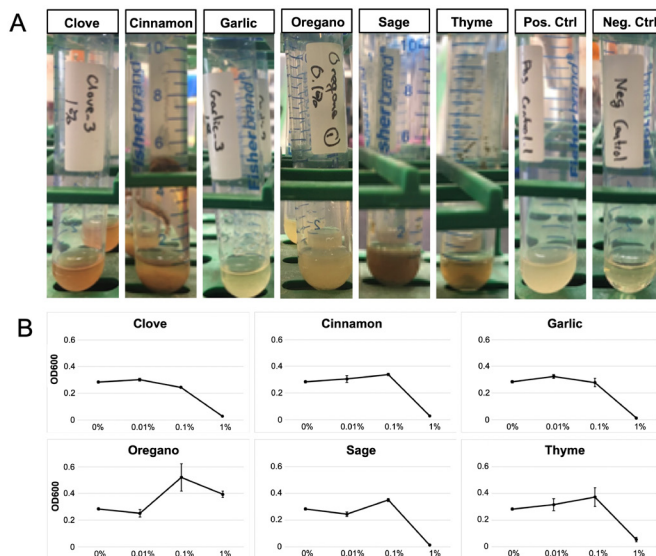


Figure 4: Dose response curves of *E. coli* grown in LB with various concentrations of spices. A) Images from *E. coli* cultures treated with 1% of six spices that demonstrated antibacterial activity in the previous experiment. B) OD600 values of *E. coli* cultures treated with 0%, 0.01%, 0.1%, and 1% of each spice. Error bars represent SEM, n=3 cultures per treatment.

Another possible counter to antibiotic resistance is the use of antibiotic adjuvants, which on their own have no antibiotic use but are able to enhance those of antibiotics when combined as part of a drug therapy (4).

Several of our results were surprising to us, including the atypical growths seen in our basil LB agar cultures, and the lack of more nuanced spice dose-dependence of our LB culture dilutions. One explanation for the atypical basil culture growths is that the dried basil was contaminated with some other bacteria or fungus prior to dilution in the agar plates. To assess whether this result was consistent with basil from a different source, we might have tested dried basil from another store brand, or even tested fresh basil. Although basil was the only spice that demonstrated these atypical growths, it is possible that contamination from other microbes might also have influenced our results. Future studies should explore autoclaving the spices, or otherwise extracting active compounds from the spices before testing. For the second experiment with the liquid LB cultures, an alternative method might have been to wait 24 hours instead of just 18 hours of incubation, as it is possible that the spices slowed bacteria growth, rather than killing bacteria entirely. This may have allowed us more accurate distinction between spices at 1% concentration because some OD600 values were too low to determine if bacteria were present. If we were to repeat this experiment, we would also test intermediate spice concentrations between 0.1% and 1%, in hopes of establishing a more detailed dose-dependence curve.

Future studies should test different spices or herbs,

beyond what we tested, to widen the knowledge of effective plant metabolites against bacteria. Follow-up experiments should also attempt different methods of extraction to concentrate the active compounds and test them independently against *E. coli*. When a compound is identified and isolated, we could use a modern antibiotic in the liquid culture and compare how effective it was at low concentration to active compounds identified from the spices and herbs. Another experiment would be to use a different bacteria strain and see if the most effective spices and herbs are also potent against other gram-negative bacteria, or even gram-positive bacterial species. Lastly, we could test if *E. coli* might eventually develop resistance to a specific spice or herb, and how long would it take to do so.

MATERIALS AND METHODS

Preparation of herbs and spices

First, we ground the dried spices and herbs (purchased from Market Basket) using a coffee grinder until they were fine in quality. For those spices already ground to a fine consistency in their packaging, such as cinnamon and black pepper, we did nothing. Then, we put all spices in labeled falcon tubes to be stored as we conducted the experiment.

Preparation of LB agar plates

To make LB agar plates with 5% spice dilution, we melted 1000 mL of an LB agar stock (FisherSci) in a microwave until boiling. While we waited for the melted LB agar to cool, we labeled 39 petri dishes with the name of spice/positive control/negative control and trial number. We then poured 60 mL of the LB agar into secondary container with 3 g of the spice (5% concentration), mixed the LB agar - spice suspension, and poured 20 mL into each of three replicate 10 cm petri dishes. The control plates were poured with just the LB media agar by itself. We let the LB agar in petri dishes solidify overnight.

Inoculation and incubation of LB agar plates

To grow a stock of *E. coli* cells, DH5- α *E. coli* (New England Biolabs) cells were thawed at room temperature, and 5 μ L were pipetted into 2 mL of LB media in a test tube. The *E. coli* were then incubated overnight at 37°C and 220 revolutions per minute. The next day, we pipetted 2 μ L *E. coli* into a NanoDrop spectrophotometer to measure number of bacteria. Then, we diluted the *E. coli* cultures with LB media to produce an estimated 2.0×10^4 CFU per mL concentration. We pipetted 20 μ L of the *E. coli* solution into the center of each plate, and spread the *E. coli* evenly over each plate using sterile glass plating beads. Last, we incubated the plates overnight at 37°C and checked their growth the next day.

Colony counting and analysis

For the analysis, we took representative images spanning 308.5 mm² of the plates under a dissection microscope (Zeiss AxioZoom). Using the Fiji application, we manually counted the number of bacteria on the images of the spices that

had visible growth of bacteria on each of their three plates. Additionally, we took a picture of one individual representative colony from each spice treatment. We used the Fiji application to measure the area of the representative colony.

Inoculation and incubation of LB liquid cultures

First, we measured out 10 mL of LB into 39 Falcon tubes (3 tubes for each condition). Next, we measured out 100 mg, 10 mg, and 1 mg for each spice (1%, 0.1%, and 0.01%). We mixed the spice with the LB media and vortexed it for 1 minute. Then, we pipetted 2 mL from each Falcon tube into culture tubes with 2000 CFU (1000 CFU/ μ L). Finally, we incubated the tubes for 18 hours at 37°C and 220 revolutions per minute.

Optical density readings and analysis

The following day, we centrifuged the tubes at 10 xG for 1 minute to pellet small spice particulate at the bottom of each tube, and collected 1 mL of the supernatant into new Eppendorf tubes with no spice particles. Then, we centrifuged the supernatant at 16000 xG for 5 minutes to pellet all *E. coli* bacteria. We poured out the remaining liquid in the Eppendorf tubes, leaving only the *E. coli* pellet, and resuspended the pellet in 1 mL Phosphate Buffered Saline (PBS).

Subsequently, we measured 2 μ L of the resuspension onto a NanoDrop spectrophotometer and recorded the OD600 value for each sample. Then, we used an online calculator to estimate CFU from each sample. After that, we found the mean and standard error for three trials of each treatment condition. Data analysis and statistics were performed on the raw OD600 values using Microsoft Excel.

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