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Antibacterial Activity of Plant-based Phenolic Compounds, Curcumin and Carvacrol

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ABSTRACT:

With the ever-increasing antibiotic resistance in the medical field, there is a need now more than ever to develop natural antibacterial alternatives. In order to address this growing concern, the natural compound Curcumin and Carvacrol were investigated against Escherichia coli (E. coli), Bacillus subtilis (B. subtilis), Staphylococcus epidermidis (S. epidermidis), and Pseudomonas aeruginosa (P. aeruginosa). Curcumin, which is an active ingredient in the well-known Turmeric plant, has been shown to have antioxidant, anti-inflammatory, and anti-microbial properties. Carvacrol, which is found in Oregano and other essential oils, has shown antimicrobial properties on a wide range of bacterial species. E. coli is a Gram-negative rod-shaped bacterium that is known to cause many bacterial infections that include urinary tract infection (UTI), cholecystitis, bacteremia, cholangitis, and other clinical infections such as pneumonia as well. B. subtilis, which is a Gram-positive bacterium, can also cause infections such as bacteremia and pneumonia but also endocarditis and septicemia. Gram-negative P. aeruginosa can cause diseases such as respiratory tract infections, UTIs, and gastrointestinal infections. Gram-positive S. epidermidis is a bacterium that can cause diseases such as endocarditis and dermatitis. The aim of this study is to investigate the antimicrobial activity of curcumin and carvacrol against these bacteria species using microplate assay and colony-forming unit (CFU) assays. The growth monitoring results indicated that 100 µg/mL of curcumin and 0.1% of carvacrol significantly inhibited four bacterial species. The cell number data obtained from the fluorescent microscopy indicated that curcumin could reach 3.26, 1.75, 3.17, and 1.65 log reduction in S. epidermidis, P. aeruginosa, E. coli, and B. subtilis, respectively. Furthermore, synergistic bacterial effect of curcumin and carvacrol with antibiotics with differing modes of action were assessed. The disc-diffusion assay results suggested no significant antibacterial synergism was observed for either curcumin or carvacrol with the selected four antibiotics.

1. Introduction

Antibiotics have been a cornerstone of modern medicine, effectively treating bacterial infections for decades. However, the extensive use and, at times, the misuse of antibiotics have given rise to a significant challenge in their own right – antibiotic resistance. This phenomenon occurs when bacteria, which were previously susceptible to the effects of antibiotics, evolve mechanisms to withstand their impact. The consequences of antibiotic resistance extend beyond individual cases; they pose a grave public health threat by compromising the effectiveness of antibiotics, making it increasingly challenging to combat bacterial diseases [1]. The emergence of antibiotic-resistant infections has become a pressing concern in the medical field. Notably, Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant Enterococci (VRE) have gained notoriety as formidable antibiotic-resistant bacterial pathogens. In 2019, the Centers for Disease Control and Prevention (CDC) classified these pathogens as "Serious Threats," highlighting the urgency of addressing this growing problem [2]. With the ever-growing problem of antibiotic resistance and antibiotic-resistant infections, there comes an increased need for alternatives to help decrease and mitigate the

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effects of resistance. As such, increasing research has been conducted into natural alternatives for antibiotics. This study aims to investigate the antibacterial properties of two natural compounds, curcumin and carvacrol, against Gram-negative and Gram-positive bacterial species.

Curcumin is a bright yellow chemical derived from the plants of the *Curcuma longa* species, more commonly known as turmeric. Turmeric is sold as a food ingredient, is used for food flavoring, and is used for food coloring as well. The molecular weight of curcumin is 368.38 g/mol. It is known to exhibit antimicrobial activity against a wide range of bacterial species. Furthermore, previous research indicates that curcumin possesses anti-inflammatory, antioxidant, antibacterial, and antiparasitic activity [3,4].

Carvacrol, on the other hand, is a natural monoterpene phenol that is commonly found in Oregano and other essential oils. It has a molecular weight of 150.22 g/mol and is reported to exhibit antimicrobial activity on a broad range of bacterial species. Additionally, it has been reported to exhibit antioxidative, anti-inflammatory, anticarcinogenic, and analgesic properties [5-7]. Similar to curcumin, carvacrol's applications include its use as a food additive and preservative [8].

Both curcumin and carvacrol are classified as 'Generally Recognized as Safe' (GRAS) substances by the U.S. Food and Drug Administration (U.S. FDA) [9]. Prior clinical trials have shown that even high doses of curcumin (12 g/ day) are shown to be safe [10]. Additionally, a clinical trial conducted on healthy subjects demonstrated the safety and tolerability of carvacrol at a dosage of 2 mg/kg/day [11]. These two compounds have not been limited to the realm of theoretical research but have found practical applications in various fields, such as food packaging and preservation, as well as medical and dental applications [12-15]. As such, their potential synergistic effect is an area of interest to determine if their antimicrobial properties can be enhanced in combination.

To examine the effectiveness of curcumin and carvacrol as antibacterial agents, an *in vitro* study has been initiated, targeting four model organisms: *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Staphylococcus epidermidis* (*S. epidermidis*). *E. coli* is a Gram-negative bacterium that can cause urinary tract infections, cholecystitis, and pneumonia [3]. *B. subtilis* is a Gram-positive species that causes pneumonia, endocarditis, and bacteremia [16]. *P. aeruginosa*, which is a Gram-negative bacterium is known to cause diseases such as respiratory tract, urinary tract, and gastrointestinal infections [17]. *S. epidermidis* is a Gram-positive bacterium which can cause dermatitis and endocarditis [18].

In summary, the issue of antibiotic resistance poses a formidable challenge to the field of medicine. The search for alternatives to traditional antibiotics has led to the investigation of natural compounds like curcumin and carvacrol. These compounds, with their proven safety and diverse properties, hold great promise. The ongoing research study on these compounds aims to shed light on their potential as antibacterial agents against various bacterial species. The study is particularly pertinent, given the safety profile of these compounds and their multifaceted applications in different fields [12-15]. This research may contribute to the much-needed arsenal of strategies to combat antibiotic resistance, thus benefiting public health.

2. Materials and methods

A. Bacterial Culture and Maintenance

Gram-negative bacteria *Escherichia coli* (*E. coli*) (155065A) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (155250A); Gram-positive bacteria *Bacillus subtilis* (*B. subtilis*) (154921A) and *Staphylococcus epidermidis* (*S. epidermidis*) (1555556A) were acquired from Carolina Biological (Carolina Biological, Burlington, NC, USA). The cultures were maintained in tryptic soy broth (TSB) (Bacto™, Sparks, MD, USA) at 37°C and routinely checked for their purity.

B. Compound Preparation

Curcumin, Curcuma longa (High Purity) was obtained from BioVision (BioVision, Milipitas, CA). The stock solution of 200 µg/mL was prepared and diluted with bacterial growth media to various concentrations tested. Purified Carvacrol (>=98%) was obtained from Sigma Aldrich Fine Chemicals Biosciences (Sigma Aldrich, St. Louis, MO, USA.

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C. Microplate-based Antibacterial Assay

Microplate-based antibacterial assay was used to monitor the growth of bacterial species with various concentrations of curcumin and carvacrol over a 24-hour period. A 20 µL of overnight culture (OD_{600nm} = ~1.0) with various concentrations of curcumin and carvacrol were added to each well of a 96-well plate. The optical density was recorded using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, USA). All experiments were done in triplicate with the mean and standard deviation calculated for statistical significance.

D. Colony-Forming Unit (CFU) Assay

Cells treated with Microplate-based antibacterial assay with 50 and 100 µg/mL curcumin and 0.05 and 0.10% for 8-hour were harvested and plated on tryptic soy agar (TSA) and incubated at 37°C for 12 hours to evaluate their viability.

E. Fluorescent Microscopy

Control and treated bacterial cells were further analyzed by fluorescence microscopy. Samples were stained with QUANTOMTM Total staining Dye following the manufacturer's protocol (Logos Biosystem, Anyang, South Korea). The stained samples were visualized with QUANTOM Tx™ Microbial Cell Counter (excitation 496 nm, emission 520 nm) to analyze their morphological differences.

F. Disc Diffusion Assay

A 10 µL of 100 µg/mL curcumin or 10 µL of 0.1% carvacrol were added to different antibiotics, ampicillin 10 µg (AM10), erythromycin 15 µg (E15), rifampicin 5 µg (RA5), and tetracycline 30 µg (TE30) respectively and incubated overnight to measure the zone of inhibition (ZOI) in millimeter (mm).

3. Results

A. Bacterial Growth Analysis with Curcumin and Carvacrol

Microplate-based antibacterial assay results indicated that 100 µg/mL curcumin was able to significant inhibit the growth of all the bacteria tested except for *S. epidermidis* in this study (Fig. 1). As for carvacrol, 0.05% carvacrol was able to significantly inhibit the growth of *E. coli* and *B. subtilis* while the MIC for *P. aeruginosa* and *S. epidermidis* was shown to be greater than 0.1% (Fig. 2).

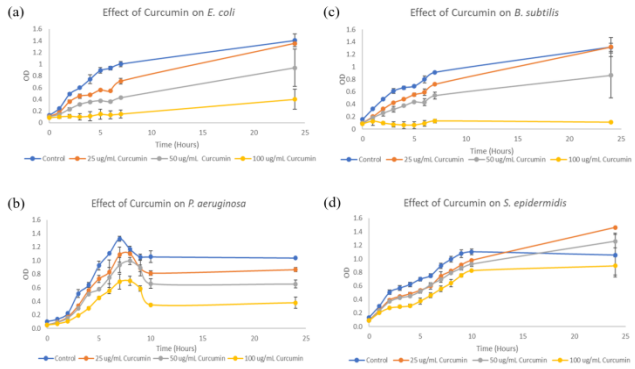

Fig. 1. Growth curve of four bacteria with 0 (blue), 25 (orange), 50 (gray), and 100 µg/mL (yellow) curcumin over a 24-hour period. (a) E. coli, (b) P. aeruginosa, (c) B. subtilis, (d) S. epidermidis.

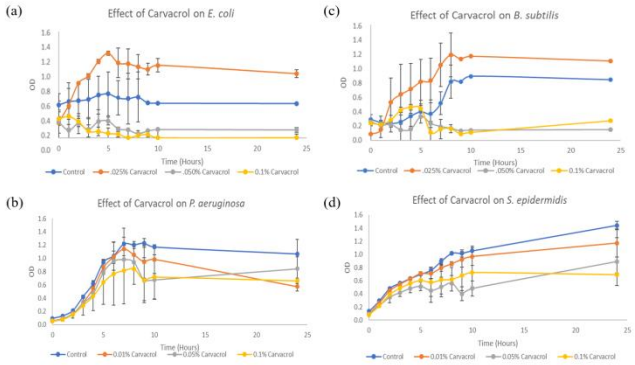

Fig. 2. Growth curve of four bacteria with 0 (blue), 0.025 (orange), 0.050 (gray), and 0.10% (yellow) carvacrol over a 24-hour period. (a) *E. coli*, (b) *P. aeruginosa*, (c) B. subtilis, (d) S. epidermidis.

B. Percent Inhibition via Colony-forming Unit (CFU) Assay

Colony-forming unit assay results obtained after the 8-hour treatment with 50 and 100 µg/mL curcumin, 0,05% and 0.10% carvacrol were analyzed to determine the percent inhibition for each bacterium. For *E. coli*, 50 and 100 µg/mL curcumin was able to inhibit bacterial growth from 98.67% and 99.93%; 89% and 98% for *P. aeruginosa*; 83.33% and 97.78% for *B. subtilis*, while 20% and 80% for *S. epidermidis*, respectively. The result was consistent from the growth analysis where

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curcumin was least effective on *S. epidermidis*. However, 0.1% carvacrol was able to inhibit *S. epidermidis* up to 97%, unlike what was seen from the microplate-based assay.

C. Fluorescent Microscopy

To further examine the antibacterial mechanisms, fluorescent microscopy analysis was carried out. Fig. 3 depicts the comparison of *P. aeruginosa* with no treatment (control) (Fig. 3a), 100 µg/mL curcumin (Fig. 3b), and 0.1% carvacrol treatment (Fig. 3c) while Fig. 4 showcases *S. epidermidis* with no treatment (control) (Fig. 4a), 100 µg/mL curcumin (Fig. 4b), and 0.1% carvacrol treatment (Fig. 4c).

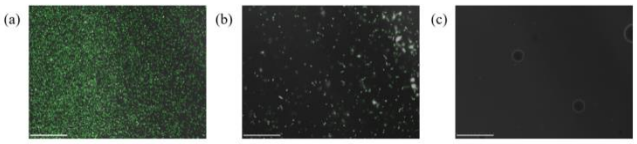

Fig. 3. Fluorescent microscopic image of *P. aeruginosa* under (a) control, (b) 100 µg/mL curcumin treatment, (c) 0.1% carvacrol treatment. Scale bar = 100 µm.

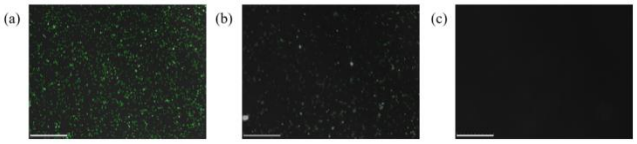

Fig. 4. Fluorescent microscopic image of *S. epidermidis* under (a) control, (b) 100 µg/mL curcumin treatment, (c) 0.1% carvacrol treatment. Scale bar = 100 µm.

The cell number obtained from fluorescent microscopy showed significant inhibition of *P. aeruginosa* and *S. epidermidis* with log reductions of 1.65-3.26 for curcumin and 1.30-2.36 for carvacrol. In addition, the cell size was also measured under each condition and the average size was calculated. The cell size of 100 µg/mL curcumin treated *S. epidermidis* (3.2 µm) was similar to the control (3.3 µm) while 0.1% carvacrol treated cells exhibited a much smaller size (0.7 µm). The cell size of 100 µg/mL curcumin treated *P. aeruginosa* (2.6 µm) was slightly smaller than the control (3.1 µm) and 0.1% carvacrol treated cells (1.7 µm) is about half the size compared with control.

D. Synergistic Antibacterial Activity Evaluation

Disc Diffusion Assay results indicated that the antibiotics. Among four antibiotics (ampicillin-AM10, erythromycin-E15, rifampicin-RA5, and tetracycline-TE30) tested, only 100 µg/mL curcumin showed 77.78% increase on the zone of inhibition (ZOI) when coupled with TE30 on *B. subtilis*. The rest of the combination showed little to no synergism when combined with either curcumin or carvacrol.

4. Conclusion and Discussion

In the realm of microbiology and antimicrobial research, our in-depth investigation aimed to unravel the antimicrobial prowess of two natural compounds, curcumin and carvacrol, against a myriad of bacterial species encompassing both Gram-negative and Gram-positive classifications. As we embark on this extended exploration, we delve into the nuances of our findings and their implications for the field, supported by relevant citations. We also explore the broader landscape of the antibacterial activity of curcumin and carvacrol, shedding light on their potential as natural alternatives to traditional antibiotics and discussing the challenges and future research directions in this evolving field.

The results obtained from our study have unveiled intriguing insights into the minimum inhibitory concentration (MIC) of curcumin and carvacrol. We recently found that 100 ug/mL curcumin was shown to be the MIC for B. subtilis. Similarly, many of the bacteria tested in this study have been tested with curcumin before and have yielded different MIC values. The reason for this discrepancy between MIC values might be due to the fact that curcumin as a compound varies greatly in purity depending on its source [19]. This variation in curcumin's purity emphasizes the necessity for future studies to diligently evaluate concentrations exceeding 100 µg/mL for curcumin and 0.1% for carvacrol. In our quest for a more comprehensive understanding, the evaluation of colony-forming units (CFU) has provided further insight on the impact of these compounds. Notably, the results showed that 100 µg/mL curcumin was able to inhibit growth at levels consistently over 80% for the bacterial species tested. Both the 0.05% and 0.1% carvacrol concentrations were able to inhibit growth levels over 97% for S. epidermidis. This assay gave a better sense

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of the effect of both compounds as it accounted for live cells only as opposed to the microplate assay which is unable to discriminate between live and dead cells. In concert with these findings, fluorescence microscopy imaging analysis offered both a qualitative and quantitative perspective on the inhibition of cell growth. The images produced clearly depict a noticeable reduction in fluorescence intensity when curcumin and carvacrol were administered at concentrations of 100 µg/mL and 0.1%, respectively. Additionally, a discernible decrease in cell size was observed, further emphasizing the compounds' impact on bacterial growth. Carvacrol, in particular, showcased a more pronounced reduction in cell size, which could indicate its potential mechanism in inhibiting bacterial replication and division. By decreasing cell size, the compound may be able to affect the ability for the bacterial cells to replicate and divide. In another study, the mechanism of carvacrol inhibiting bacterial species suggested its lipophilic properties bind to the bacteria's cell membrane, decreasing the membrane potential, ion exchange, and energy production of the cell [20]. These factors increased cellular ROS levels, suggesting a mechanism of how protein oxidation, DNA damage, and cell death followed. As we contemplate the future of antibacterial research, it is essential to consider the wider landscape. Curcumin, in particular, has garnered attention for its direct broad-spectrum antibacterial activities against both Gram-negative and Gram-positive bacteria. This versatile compound also serves as an immunomodulator, where it boosts bacterial infections by blocking the pathogen's virulence factors and augmenting host-mediated immunity.

According to a review of previous studies on curcumin, a diverse mechanism of action was attributed to different bacteria species. For E. coli and P. aeruginosa, potential mechanisms included alteration of the membrane permeability and disruption in biofilm structure [21]. For S. epidermidis and B. subtilis, a potential mechanism included decrease biofilm formation [22].

Based on the results obtained, it appears that both compounds are effective natural alternatives to antibiotics, however, further studies need to be done to confirm these results. Research into additional bacterial species would provide a greater understanding of their effectiveness across the board. Additionally, the potential synergistic effect in combination with four selected antibiotics (data not shown), but no significant results were obtained. As such, it would be beneficial to further investigate the combination of these compounds with existing antibiotics to determine if they can be combined to create a synergistic effect. Further studies utilizing these two compounds may involve the possibility of their synergistic effect in combination with one another.

This study evaluated the effects of curcumin and carvacrol on two Gram-positive and two Gram-negative bacteria. Microplate and CFU assays were conducted which indicated that the MIC values for curcumin and carvacrol were greater than 100 µg/mL and 0.1%, respectively. Fluorescence imaging analysis was carried out to visualize and quantify the changes in cell concentration and size for S. epidermidis and P. aeruginosa. This analysis showed a decrease in cell concentration with the two compounds compared to the control as well as a decrease in cell size.

Curcumin has been shown to possess direct broad-spectrum antibacterial activities against Gram-negative and Gram-positive bacteria. It also acts as an immunomodulator whereby it ameliorates bacterial infections by blocking the pathogen's virulence factors and augmenting host-mediated immunity. Curcumin has a marked synergistic or additive anti-bacterial activity in combination with some traditional antibacterial drugs and natural active substances. Animal studies have also demonstrated curcumin's efficacy as a treatment for localized trauma-caused skin infections, underscoring its practicality in various medical contexts. Importantly, human trials have shown that oral administration of curcumin is both safe and effective in managing a range of skin diseases, including psoriasis, infection, acne, skin inflammation, and skin cancer. Nonetheless, it is crucial to acknowledge the existing challenges associated with curcumin as a potential antibacterial therapy. These challenges include the inadequately understood critical targets and precise molecular mechanisms, poor solubility, low bioavailability, and rapid degradation in humans or animals when consumed orally, and no effective clinical trials [23].

In the quest to harness the full potential of curcumin and carvacrol, it is crucial to explore innovative avenues. Curcumin, for instance, has the potential to be

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used as a broad-spectrum antibacterial adjuvant to permeabilize the bacterial membrane. Additionally, it exhibits potential as a novel strategy for treating thyroid cancer. Scientists have developed various curcumin nano-formulations, which have exhibited better solubility and antibacterial activity compared to native curcumin. However, there is a lack of evidence-based randomized investigation especially exploring the therapeutic roles of the nanocarrier-based delivery systems in enhancing anti-bacterial actions; therefore, much needs to be explored. In addition, other types of new formulations (e.g., inclusion technology, solid dispersion technology, microspheres, and microcapsules) were also developed to improve the solubility and bioavailability of curcumin. Further research is needed to explore the potential of curcumin as an antibacterial agent and to develop effective delivery systems for curcumin.

Carvacrol, on the other hand, exhibits considerable potential in the food packaging industry due to its antimicrobial properties. It has demonstrated efficacy against a broad spectrum of bacteria, including a variety of food-borne and medically important pathogens. Additionally, the synergic antimicrobial effects between carvacrol and various antimicrobial agents have been observed which offer an exciting avenue for research. These compounds hold promise in inhibiting microbial spoilage in food products and serving as potent antimicrobial agents against antibiotic-resistant bacteria [24]. However, it is important to note that confirming these activities requires real-world testing on actual food products. This is due to the increased complexity of microbial flora and the matrix of foods, which may necessitate higher doses of these compounds to achieve considerable biological activity [24].

Nanotechnology has generated considerable effects on medical sciences and the food packaging industry, and the potential ability of nanoparticles has been studied in the management of infections and control of food-borne pathogens. Different nanoparticle-based delivery approaches, particularly liposomes, polymers, carbon nanotubes, and inorganic nanomaterials, have received increasing attention. These approaches offer an improved pharmacokinetic pattern and therapeutic effects when drug molecules are incorporated into nano-delivery systems. This technology opens the door to exploring the use of nanoformulations of carvacrol as more effective and targeted strategies for combating bacterial infections and food spoilage [24]. In conclusion, our extensive study has provided invaluable insights into the antimicrobial activities of curcumin and carvacrol, showcasing their potential as natural alternatives to conventional antibiotics. However, it is clear that further research is necessary to confirm and expand upon these findings, as well as to overcome the existing challenges.

Future studies should focus on examining the potential synergistic effects of these compounds with antibiotics, delving into the intricate molecular mechanisms involved, and addressing issues related to solubility and bioavailability. Additionally, the exploration of novel delivery systems, including nanotechnology, offers exciting avenues for enhancing the antibacterial properties of these natural compounds.

The potential of curcumin and carvacrol extends beyond laboratory studies, with practical applications in the fields of medicine, food preservation, and antimicrobial therapy. By embracing these challenges and exploring new horizons, we can harness the full potential of these natural compounds to combat bacterial infections and ensure food safety. This evolving field promises a brighter future in the fight against microbial threats.

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