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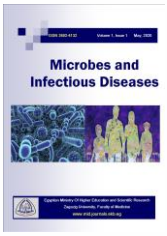

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Original article

Antimicrobial combination effect of allicin, lycopene and quercetin on antibiotic-resistant bacteria

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ABSTRACT

Background: The application of phytochemicals to treating microbial infections in humans globally is increasing with potent activity in combination. **Aim:** The study evaluated the antimicrobial combination effect of allicin, lycopene, and quercetin extracted from garlic, tomatoes, and onions respectively against methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE) and multi-drug resistant (MDR) *Escherichia coli*. **Methods**: Six antibiotic-resistant isolates: three clinical isolates and three American isolates were tested. Antimicrobial combination effect of the extracted phytochemicals was evaluated using the agar well diffusion method at varied concentrations and incubation periods. **Results**: At 50 µg, quercetin inhibited MDR *Escherichia coli* and MRSA alone with mean zones of 15±0.95 mm – 49±3.09 mm whereas lycopene inhibited MRSE with the highest mean zone of 19±1.20 mm. Combination activity of allicin: lycopene: quercetin was best at 2:3:2 µg at both 24 h and 48 h with mean zone 20±1.26 mm – 44±2.77 mm and at 5:1 µg concentration for lycopene: quercetin with mean zone 16±1.01 mm - 25±1.58 mm for all test bacteria. On the typed culture isolates, the inhibition mean zones were 8±0.50 mm – 20±1.27 mm and 8±0.50 mm – 22±1.39 mm for singly and in combination, respectively. There was a significant ($p < 0.05$) combination activity of the phytochemicals on the test bacteria at 24 h and 48 h. **Conclusion:** To our knowledge, this is the first report of the antimicrobial combination activity study of allicin, lycopene, and quercetin. Our findings suggest further combination evaluations of phytochemicals for effectively controlling antibiotic-resistant infections.

Introduction

Antibiotic-resistant microbial infections have continuously threatened public health. Globally, these infections are causing deaths and have an increasing incidence [1]. With the constant evolution of antimicrobial resistance, these pathogenic bacteria have rendered existing antibacterial less effective and even ineffective in some cases. The World Health Organization has enlisted antibiotic-resistant pathogens based on their level of threat of which multi-drug-resistant *Escherichia coli* and methicillin-resistant *Staphylococcus* species are described as critical and high-priority pathogens respectively [2]. Therefore,

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a global problem, and as a result, new antimicrobials are required urgently. Pathogens are classified as either multidrug-resistant (MDR) or extensively drug-resistant (XDR) by the Centers for Disease Control and Prevention (CDC) [3] depending on their resistance profile. Methicillin-resistant *Staphylococcus* species (MRSS) and MDR Escherichia coli are antibiotic-resistant pathogens that are typically associated with nosocomial infections with high mortality and morbidity rates, and they are responsible for a wide range of infections worldwide. Few longitudinal cohort studies in Nigeria and around the world on MDR infections in patients infected with methicillin-resistant *Staphylococcus aureus* (MRSA) had a 32 - 50% associated mortality rate [4-7]. Several other studies have reported a similar mortality rate for MDR *Escherichia coli* (*E. coli*) [8, 9].

Phytochemicals are compounds found in plants with medicinal properties to treat chronic diseases and prevent infections and neurodegenerative diseases applied in many regions of the world. These bioactive compounds can be found via extraction from plants or food sources [10]. In recent times, the combination of these compounds with a potential increase in antibacterial properties poses a novel therapeutic approach against bacterial resistance [11]. Phytochemical combinations have proven promising as resistance-modifying agents demonstrate diverse mechanisms of action. Phytochemical antimicrobial synergist's activity involves inhibition of active site modification, inhibiting enzymes that degrade and/or modify drugs, and inhibiting permeability enhancers and/or efflux pumps [12]. Allicin (organosulphur compound) isolated from garlic (Allium sativum) remains one of the most common phytochemicals studied for their antimicrobial combination effect [13]. Its antibacterial activities have been observed against a wide range of bacteria such as *Staphylococcus epidermidis* and MRSA (oral pathogens) which are known causative agents for periodontitis [11]. There are reports that allicin may function better in combination with other antimicrobials (phytochemicals) compared to when used alone [14]. Quercetin (phenolic compound) readily isolated from onions (Allium cepa) has shown diverse mechanisms of action against different bacterial strains from antimicrobial synergistic activity ranging from the inhibition of efflux pumps, interacting with the cell membrane and inhibition of cell wall biosynthesis to the inhibition of essential enzymes such as sortase A, dihydrofolate reductase and urease [15]. Quercetin and apigenin are two abundantly present phytochemicals in onions (Allium cepa) that have been reported to demonstrate significant antimicrobial activity in combination with resulting inhibition of the *E. coli* d-alanine:d-alanine ligase enzyme [16]. Furthermore, lycopene is a carotenoid readily isolated phytochemical found in tomatoes (Solanum lycopersicum) with a report on antimicrobial activity against drug-resistant *Staphylococcus aureus* and *E. coli* alone [17] however; no report on antimicrobial combination activity.

There is a critical need for new antibacterial agents to combat the threat of antibiotic resistance. Unfortunately, the development of this trend is remarkably slow. The phytochemicals: allicin, quercetin, and lycopene have exhibited potent antimicrobial activities as natural therapeutic alternatives with diverse physiological and pharmacological responses in the body [13]. However, up to this date, the antimicrobial combination activity of these three natural compounds against antibiotic-resistant pathogenic bacteria has largely remained elusive. In the present study, we have explored the antimicrobial combination activity of allicin, quercetin, and lycopene against MDR *E. coli* and methicillin-resistant *Staphylococcus* species for enhanced antibacterial activity. This study aimed to evaluate the antimicrobial combination effect of allicin, lycopene, and quercetin against antibiotic-resistant pathogens: MRSA, MRSE, and MDR *E. coli* and American-type cultures.

Methods

Extraction of allicin, lycopene, and quercetin

Allicin, lycopene, and quercetin were extracted from garlic (Allium sativum), tomatoes (Solanum lycopersicum), and onions (Allium cepa) respectively. Fresh plant samples were cleaned with sterile distilled water and left to oven-dry at 55 ^{o}C for 6 days. Afterward, each of the plant samples was placed in a sterile grinder to produce a smooth powder.

The samples were mixed with the extraction solvent (acetone) in the ratio of 1: 10 [17] except for allicin which was done in 1:2 according to methods by **Arzanlou et al.** [18]. The mixture was steeped for 2 h at a room temperature of 27±2 °C in a mechanical shaker and then filtered. The filtrate was

concentrated using a rotary evaporator at 56 ºC for 30 minutes. The concentrate absorbance was read on a UV-Vis spectrophotometer at 272 nm, 471 nm, and 372 nm for allicin, lycopene, and quercetin respectively.

Collection of test bacteria species

Three (3) clinical bacteria isolates, namely: methicillin-resistant *Staphylococcus epidermidis*, multi-drug resistant *Escherichia coli*, and methicillin-resistant *Staphylococcus aureus (S. aureus)*, were obtained from the Microbiology Laboratory, Department of Biological Sciences and Biotechnology, Caleb University, Nigeria. The three (3) American-type cultures included: methicillin-resistant *S. aureus* ATCC 43300, *S. epidermidis* ATCC 12228, and *E. coli* ATCC 35218. Pure cultures of the bacterial isolates were obtained via sub-culturing on a nutrient agar plate and incubated at 37 °C for 24 h. The pure isolates were further subjected to standard microbiological procedures: gram-staining, and biochemical analysis.

Phytochemical inhibition test

The Phytochemical inhibition test was performed alone and in combination with the six bacteria species in triplicates using the agar well diffusion method. On Muller-Hinton agar, wells of 6 mm were bored after inoculation of each test bacteria of 1.5 × 108 CFU/mL suspension. Varied concentrations: 10 µg, 20 µg, 30 µg, 40 µg and 50 µg were dispensed for each phytochemical into the well. Whereas, for dual combination testing; lycopene: quercetin was dispensed in five (5) different concentrations: 1:3 µg, 3:1 µg, 1:5 µg, 5:1 µg and 1:2 µg. For triple combination testing, allicin: lycopene: quercetin was similarly dispensed in five different concentrations: 2:3:2 µg, 3:3:1 µg, 3:1:1 µg, 1:1:1 µg, and 1:3:1 µg.

The inoculated plates were incubated for 2 h at room temperature (to aid proper diffusion of the phytochemicals into the medium) before further incubation at 37 °C for 24 h and 48 h. Bacterial inhibition was determined by the extent of the zones of inhibition.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). The statistical analysis was done via SPSS version 20 (SPSS Inc. USA). The generated data were subjected to a one-way analysis of variance (ANOVA) test to compare the mean values with significant differences ($p < 0.05$) in the incubation time and phytochemical concentration. Fisher's least significant difference (LSD) was performed for the mean pairwise comparison of the different concentration groups at a 95% level of confidence.

Results

The combined antibacterial activity of allicin, lycopene, and quercetin extracted from garlic (Allium sativum), tomatoes (Solanum lycopersicum) and onions (Allium cepa) respectively were tested on three (3) clinical antibiotic-resistant bacteria isolates: Multi-drug resistant (MDR) *Escherichia coli*, Methicillin-resistant *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus epidermidis* likewise; three (3) American type cultures: Methicillin-resistant *S. aureus* ATCC 43300, *S. epidermidis* ATCC 12228 and *E. coli* ATCC 35218. At two incubation times (24 h and 48 h), the phytochemicals were tested individually at different concentrations of 10 µg, 20 µg, 30 µg, 40 µg, and 50 µg. The three phytochemicals had an inhibitory effect on the tested bacteria across the various concentrations except for allicin at 10 µg and 20 µg tested on MDR *E. coli* **(Table 1-3).** The phytochemicals were most active against all the tested bacteria at 50 µg concentration at an incubation period of 48 h. The mean zone of inhibition was highest against MDR *E. coli* by lycopene and quercetin with an inhibition zone of 40±2.52 mm and 49±3.09 mm respectively.

Allicin was most active against *S. epidermidis* ATCC 12228 with a mean zone of inhibition of 29±1.83 mm. For MDR *E. coli*, allicin and quercetin had no inhibitory effect at 10 µg concentration. Although lycopene had an inhibitory effect at 10 µg, the mean zone of inhibition (3±0.19 mm) was significantly low at both incubation periods of 24 h and 48 h. For the methicillin-resistant *Staphylococcus* species, there was a progressive inhibitory effect by the phytochemicals across the different concentrations. Similarly, among the typed culture there was an upward increase in the inhibitory effect of the phytochemicals.

For antimicrobial combination activity, the phytochemicals were tested in dual combination and triple combination at varied concentrations and incubated for both 24 h and 48 h. For dual combination activity, the combination lycopene: quercetin was tested against all test bacteria in the concentrations of 1:3, 3:1, 1:5, 5:1, and 1:2 µg. Among the clinical isolates, the best dual

combination concentration was 5:1 µg at 48 h against MDR *E. coli* with a mean inhibition zone of 29±1.83 mm followed by methicillin-resistant *S. epidermidis* with a mean inhibition zone of 25±1.58 mm **(Table 4).** Similarly, the concentration of 5:1 µg had the best result of inhibition against *S. epidermidis* ATCC 12228 with a mean inhibition zone of 22±1.39 mm followed by methicillin-resistant *S. aureus* ATCC 43300 with a mean zone of inhibition 21±1.32 mm at 48 h.

Combination activity of allicin: lycopene: quercetin on test organisms revealed the best concentration at 2:3:2 µg at both 24h and 48 h with significant ($p < 0.05$) effect followed by 3:3:1 µg against both the clinical isolates and the typed culture **(Table 5).** The highest mean zone of inhibition was seen in MDR *E. coli* and ATCC 12228 for the clinical isolates and typed culture respectively.

Table 1. Susceptibility of the test organisms to allicin.

Organisms	**Zone of inhibition at different concentrations (mm)**									
	10 µg		20 µg		30 µg		40 µg		50 µg	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDR *E. coli*	NZ	NZ	NZ	NZ	10±0.63	14± 0.89	14± 0.89	20±1.27	21±1.32	25±1.58
MR *S. aureus*	10±0.63	13±0.82	12±0.76	15±0.95	9±0.57	15±0.95	16±1.01	26±1.64	20±1.27	25±1.58
MR *S. epidermidis*	10±0.63	13±0.82	12±0.76	15±0.95	13±0.82	16±1.01	13±0.82	17±1.07	15±0.95	20±1.27
MR *S. aureus ATCC 43300*	5±0.32	8±0.50	5±0.32	11±0.70	12±0.76	14±0.88	21±1.32	23±1.45	32±2.02	38±2.40
S. epidermidis ATCC 12228	8±0.50	10±0.63	8±0.50	13±0.82	10±0.63	15±0.95	11±0.70	15±0.95	23±1.45	29±1.83
E. coli ATCC 35218	8±0.50	10±0.63	8±0.50	10±0.63	10±0.63	12 0.76	11±0.70	11±0.70	12±0.76	14±0.88

KEY: NZ-No zone of inhibition; MDR-Multi drug-resistant; MR: Methicillin-resistant

Table 2. Susceptibility of the test organisms to lycopene

Organisms	**Zone of inhibition at different concentrations (mm)**									
	10 µg		20 µg		30 µg		40 µg		50 µg	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDR *E .coli*	3±0.19	3±0.19	5±0.32	5±0.32	12±0.76	14±0.88	21±1.32	23±1.45	37±2.33	40± 2.52
MR *S. aureus*	8±0.50	10±0.63	8±0.50	13±0.82	10±0.63	15±0.95	11±0.69	15±0.95	13±0.82	19±1.20
MR *S. epidermidis*	10± 0.63	11±0.69	12±0.76	13±0.82	13±0.82	18±1.13	13±0.82	19±1.20	15±0.95	19±1.20
MR *S. aureus ATCC 43300*	8±0.50	10±0.63	8±0.50	10±0.63	10±0.63	12±0.76	11±0.69	11±0.69	12±0.76	14±0.88
S. epidermidis ATCC 12228	5±0.32	8±0.50	8±0.50	12±0.76	8±0.50	13±0.82	9±0.57	13±0.82	10±0.63	14±0.88
E. coli ATCC 35218	11±0.69	13±0.82	15±0.95	18±1.13	13±0.82	21±1.32	15±0.95	25±1.58	23±1.45	33±2.08

KEY: MDR-Multi drug-resistant; MR: Methicillin-resistant

Table 3. Susceptibility of the test organisms to quercetin

Organisms	**Zone of inhibition at different concentrations (mm)**									
	10 µg		20 µg		30 µg		40 µg		50 µg	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDR *E. coli*	NZ	NZ	10±0.63	10±0.63	11±0.69	13±0.82	28±1.76	38±2.40	40±2.52	49±3.09
MR *S. aureus*	10±0.63	13± 0.82	12±0.76	15±0.95	13±0.82	16±1.01	13± 0.82	17± 1.07	15± 0.95	20± 1.27
MR *S. epidermidis*	10±0.63	15±0.95	11±0.69	16±1.01	11±0.69	19±1.20	13± 0.82	20± 1.27	14± 0.88	20± 1.27
MR *S. aureus ATCC 43300*	10± 0.63	13±0.82	12±0.76	15±0.95	13±0.82	15±0.95	13± 0.82	16± 1.01	15± 0.95	20± 1.27
S. epidermidis ATCC 12228	8± 0.50	10±0.63	9± 0.57	14±0.88	10±0.63	14±0.88	12± 0.76	15± 0.95	13± 0.82	16± 1.01
E. coli ATCC 35218	13± 0.82	16±1.01	15±0.95	18±1.13	21±1.32	23±1.45	25± 1.58	25± 1.58	20± 1.26	27± 1.70

KEY: NZ-No zone of inhibition; MDR-Multi drug-resistant; MR: Methicillin-resistant

Table 4. Combination activity of lycopene: quercetin on test organisms

Organisms	**Zone of inhibition at different concentrations (mm)**									
	1:3 µg		3:1 µg		1:5 µg		5:1 µg		1:2 µg	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
MDR *E. coli*	12 ± 0.76	17 ± 1.07	10±0.63	15±0.95	16±1.01	24±1.51	26 ±1.64	29 ±1.83	7±0.44	18 ±1.13
MR *S. aureus*	10 ±0.63	13±0.82	13±0.82	18±1.13	15±0.95	18±1.13	16±1.01	23±1.45	8±0.50	12±0.76
MR *S. epidermidis*	11±0.69	16±1.01	13±0.82	20±1.26	15±0.95	21±1.32	18±1.13	25±1.58	10±0.63	14±0.88
MR *S. aureus ATCC 43300*	10±0.63	13±0.82	11±0.69	15±0.95	12±0.76	15±0.95	15±0.95	21±1.32	8±0.50	12±0.76
S. epidermidis ATCC 12228	10±0.63	14±0.88	9±0.57	15±0.95	12±0.76	16±1.01	13±0.82	22±1.39	8±0.50	13±0.82
E. coli ATCC 35218	10±0.63	14±0.88	14±0.88	11±0.69	21±1.32	27±1.70	18±1.13	20±1.27	11±0.69	13±0.82

KEY: MDR-Multi drug-resistant; MR: Methicillin-resistant

Table 5. Combination activity of allicin: lycopene: quercetin on test organisms

Organisms	**Zone of inhibition at different concentrations (mm)**										*P*-value
	1:1:1 µg		1:3:1 µg		3:1:1 µg		3:3:1 µg		2:3:2 µg		
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	
MDR *E. coli*	10±0.63	10±0.63	12±0.76	15±0.95	14± 0.88	22±1.39	21±1.32	40±2.52	30±1.89	44±2.77	0.031
MR *S. aureus*	13±0.82	17±1.07	13±0.82	16±1.01	10± 0.63	19±1.20	23±1.45	28±1.76	26±1.64	35±2.21	0.016
MR *S. epidermidis*	10±0.63	15±0.95	11±0.69	16±1.01	15± 0.95	20±1.26	19±1.20	25±1.58	24±1.51	30±1.89	0.023
MR *S. aureus ATCC 43300*	10±0.63	13±0.82	12±0.76	15±0.95	10± 0.63	15±0.95	16±1.01	26±1.64	20±1.26	25±1.58	0.043
S. epidermidis ATCC 12228	12±0.76	15±0.95	14±0.88	19±1.20	13± 0.82	21± 1.32	22± 1.39	25±1.58	23±1.45	31±1.95	0.011
E. coli ATCC 35218	11±0.69	19±1.20	15±0.95	20±1.26	21± 1.32	23± 1.45	19± 1.20	26±1.64	20±1.26	27±1.70	0.037

MDR-Multi drug-resistant; MR: Methicillin-resistant

Discussion

For decades, plants have contributed to the advancement of human health as regards disease prevention and treatment globally [19]. Studies on phytochemicals have birthed the discovery of new antimicrobials with potential applications in biomedicine. Although there are reports on the antimicrobial efficacy of allicin, lycopene, and quercetin; the combination effect of the three phytochemicals in antimicrobial resistance is limited.

The resistance to allicin and quercetin by MDR *E. coli* at a low concentration of 10 µg could be due to the low concentration of phytochemicals. This Gram-negative bacterium is well-known for resistance to common antibiotics: cotrimoxazole (50 µg), streptomycin (15 µg), gentamicin (10 µg), ciprofloxacin (5 µg), and erythromycin (15 µg). The cellular structure of this bacterium with the presence of phospholipids and lipopolysaccharides as well as the presence of resistance genes could be attributed to its multi-drug resistance [20]. **Moloney** [10] reported the inhibitory activity of natural products

such as phytochemicals which can substitute for antibiotics as resistance to natural products is low. Interestingly, all the phytochemicals demonstrated inhibitory effects against all the type cultures, unlike the clinical isolates at low concentrations.

These phytochemicals can be administered at a tolerable concentration of 50 µg or higher. This corroborates studies by **Rehman et al**. [21] with phytochemicals studied at tolerable doses. These phytochemicals are known to be well-tolerated by the human body hence no report of toxicity at a high level. **Barbieri et al.** studied the antibacterial activity of allicin and quercetin against both antibiotic-resistant bacteria and non-resistant bacteria. Methicillin-resistant *S. aureus, E. coli,* and *Klebiella pneumoniae* were the antibiotic-resistant bacteria studied [16].

Bacteria are known to produce alternative target sites thus protecting them from antibiotics. This altered target confer resistance to administered antibiotics resulting in challenges in treatment. A typical example as it relates to this study is the common alteration in penicillin-binding protein by methicillin-resistant *Staphylococcus* species [11]. This altered protein is responsible for decreased affinity to the β-lactams antibiotics such as; penicillin. The efficacy at 5:1 µg by the phytochemicals could be due to the higher proportion of lycopene. Lycopene has been reported to exhibit remarkable inhibition against drug-resistant clinical bacteria isolates [17].

Amin et al. [22] reported the combination effects of phytochemicals (quercetin and allicin) extracted from species of allium against methicillin-resistant *S. aureus*; an alternative for the treatment of several infectious diseases in biomedicine. Our study further corroborates the study by **Miklasiriska-Majdanik et al.** [23] on diminished antibiotic resistance among bacteria induced by phenolic compounds as studied here.

Interestingly, onions (Allium cepa), garlic (Allium sativum), and tomatoes (Solanum lycopersicum) are plant sources that are present in daily meals. These isolated phytochemicals are biologically active and safe for consumption and are significant in traditional medicine as toxicity or poisoning is not associated with consumption. However, purely isolated allicin, lycopene, and quercetin will be best used in combination with proven medical implications.

This study was performed to evaluate the antimicrobial activities of allicin, lycopene, and quercetin in combination against antibiotic-resistant human and clinical pathogens. To our knowledge, this study provides the first investigation on the antimicrobial combination effect of the three phytochemicals- allicin, lycopene, and quercetin against antibiotic-resistant clinical and human pathogens. Moreover, allicin and quercetin had shown antibacterial combination activities on selected pathogenic bacteria species. Our results on the antimicrobial combination effect were from recent literature on the combination effect of phytochemicals.

Conclusion

The findings suggest the synergism of allicin: lycopene: quercetin phytochemicals best applied at 2:3:2 µg for effective control of antibiotic-resistant infections by methicillin-resistant *Staphylococcus* species, MDR *Escherichia coli*, and other bacterial pathogens. The combination of these isolated phytochemicals with demonstrated antimicrobial combination effect consequently; improves their activity. The study also suggests the use of phytochemicals like allicin, lycopene, and quercetin to control antibiotic resistance among clinical pathogens: methicillin-resistant *S. aureus*, methicillin-resistant *S. epidermidis*, multi-drug resistant *E. coli* and other antibiotic-resistant bacterial pathogens that pose as a threat to human health.

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Conflict of interest

The authors declare that there is no conflict of interest.

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