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Original Article

Carvacrol and Cinnamaldehyde as Next Generation Antimicrobial Agents against Foodborne Pathogens: Antibacterial Efficacy and Synergistic Interaction with Nisin

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Foodborne infections cause substantial issues all around the world, hence food safety is a major public health concern. The goal of this research is to provide information to the scientific community on the possible application of two plant essential oil molecules, Carvacrol (CAR) and Cinnamaldehyde (CN), as next-generation antimicrobials in the fight against foodborne pathogens. CAR and CN demonstrated outstanding dose-dependent antibacterial efficacy against 10 foodborne pathogens. Both Gram-positive and Gram-negative bacteria were suitably inhibited by the drugs. Antimicrobial activity of 10% v/v CAR and CN was much higher than that of the conventional antibiotic azithromycin (15g/disc). The study discovered that CAR and CN have very low MIC values (0.08% to 0.31% v/v), indicating that the compounds are efficacious even at very low concentrations. Even at 0.16% concentration, combining CAR and CN with Nisin (a widely available natural preservative) exhibited substantial synergism, with inhibition zones ranging from 8.5-12.38 mm, decreasing the dose required to produce sufficient antimicrobial action. The findings imply that CAR and CN combined with nisin can be used as a natural antibacterial agent to limit the growth of foodborne pathogens and as a natural food preservative.

Keywords: **Antimicrobial activity, Carvacrol, Cinnamaldehyde, Nisin, Foodborne pathogens, Synergistic effect**

Introduction

Consumption of foods and drinks contaminated with foodborne microorganisms causes foodborne illness, a pervasive hazard for human health. WHO reports 600 million instances of foodborne illnesses and 420,000 fatalities worldwide each year[1]. In this case, Bangladesh is no exception, and many individuals in our country still lack adequate access to necessities such as healthy food, clean and safe water, and appropriate health care. The main issue of public health is concerned with safe foods. Food poisoning is the unhygienic preparation of food and improper distribution to the consumers. Food poisoning can also be caused by environmental contamination. Antibiotics are sometimes used to treat foodborne illnesses. However, overuse of these antibiotics increases the risk of multidrug-resistant foodborne bacteria emerging. Since ancient times, many food preservation systems have been used, such as freezing, chilling, pickling, salting etc; these procedures, however, cannot protect foods from microbial attack indefinitely[2]. To protect their products from microbial contamination and extend shelf life, food manufacturers utilize a variety of chemical preservatives. However, this chemical preservative poses a significant health danger. Chemical preservatives might also be quite costly. As a result, the search for healthier food additives and preservatives to replace chemical additions has become an intriguing research subject.

Antimicrobial compounds produced from natural sources, such as plant essential oils and active components, have much potential for food preservation. New and advanced technologies have revealed the causes of these processes. The reason for this is that plant materials have antibacterial properties. Essential oils extracted from spices and herbs contain active antimicrobial compounds that indicate the possibility of using these agents in natural food preservation. Antibacterial, antiviral, antifungal, and insecticidal activities are all present in essential oils[3,4]. They also have antioxidant effects[5]. Essential oils are increasingly used in food preservation due to their unique functional qualities. Commercially available EO-based natural food preservatives are currently available, such as 'DMC Base Natural'[6] food preservatives. As a result, essential oils can be a viable alternative to artificial preservatives for food preservation.

Carvacrol (CAR) is a monoterpenoid-phenolic compound present in the essential oil of *Origanum vulgare* (oregano), thyme, pepperwort, and wild bergamot[7]. It has been reported that among phenolic compounds, CAR has one of the most potent antimicrobial activity[8]. CAR's other health benefits render potential anti-cancer activity[9], anti-inflammatory, and antioxidant[10] properties, demonstrating that it is a favorite food additive. The antimicrobial activity of CAR is mainly attributed to its membrane disrupting potential, reduction in ATP synthesis,

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dissipating cellular membrane potential, and pH gradient, affecting potassium ion efflux[11]. Carvacrol has also been reported to inhibit â-lactamase activity in *E. coli*, inducing reactive oxygen species production and cellular release of DNA, proteins and ions[12]. Cinnamaldehyde (CN), the active ingredient of cinnamon essential oil is also reported for its antimicrobial effect[13, 14]. CN alters ghrelin secretion and thus plays a role as an anti-obesity and anti-hyperglycemic factor[15]. Anticancer[16] and neuroprotective[17] role of CN has also been reported. Nisin, another antimicrobial used in this study, is a bacteriocin in nature produced by *Lactococcus lactis.* Nisin has been used in the food industry as a natural preservative for many years[18]. Its antimicrobial property can be primarily modified by combining it with other antimicrobials, which may also offer novel next-generation antimicrobials.

As minor contamination in food can cause a severe health hazard, assurances about food safety are critical concerns for the food processing industries. Chemical preservatives used for food processing have potential carcinogenic and teratogenic attributes. The development of more and more natural alternatives to chemical preservatives would solve this problem. With all of these concerns in mind, this study attempted to assess the antibacterial activity of carvacrol (CAR) and cinnamaldehyde (CN) against a variety of foodborne pathogens and spoilage microorganisms. The investigation is divided into three key sections. The antibacterial activity of CAR and CN against reference strains is determined in the first part; the MIC and MBC values of CAR are determined in the second part, and the synergistic impact of CAR and CN with nisin is determined in the third part.

Materials and Methods

Essential oils and reference strains

Carvacrol and cinnamaldehyde were the essential oils employed in this investigation (Wako Pure Chemical Industries Limited, Osaka, Japan). The antimicrobial assay employed ten strains of foodborne pathogens and spoilage bacteria as reference strains, including *Bacillus cereus, Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Salmonella typhi*, *Shigella flexneri*, *E. coli*, *Vibrio cholerae*, *Micrococcus luteus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The test organisms were received from the Department of Microbiology, University of Dhaka.

Stocking essential oil solutions

Each essential oil's crude samples were concentrated to 100% (v/v). For MIC determination, liquid essential oil was carefully measured with a measuring cylinder and diluted in 95 percent ethanol to obtain 10%, 5%, 2.5% (v/v) and 1.25%, 0.63 %, 0.31 %, 0.16 %, and 0.08 %. The stock was divided into 5ml aliquots and kept at -20º C until needed.

Filter paper discs are impregnated with stock solutions.

Fifty (50) µl of varying concentrations of substances were impregnated into 6 mm diameter nitrocellulose-filter paper discs. The solvents were evaporated by drying the discs in a hot air oven at 400°C for 1 hour, then storing them at 40°C until use. Control discs containing only ethanol but no essential oil were also made.

Assessment of the antimicrobial activity of Carvacrol and Cinnamaldehyde

The antibacterial activity of CAR and CN against the test organisms were evaluated using the Kirby-Bauer disc diffusion assay. The test organisms cultured on nutrient agar plates were inoculated on Mueller-Hinton Broth and incubated at 37ºC for 6 to 8 hours. The incubated cultures' optical density (OD.) was adjusted to obtain a suspension containing approximately 1.5×10^8 CFU/ml bacterial count. OD, adjusted cultures were inoculated on dried Muller-Hinton Agar plates using the lawn method. Essential oil-impregnated discs were dispersed over the surface of the infected agar plate with the right spatial arrangement using ethanol dipped and flamed forceps. Each disc was pushed down to establish complete contact with the agar surface. The discs were positioned with no more than 24 mm between their centers. Each plate had 4 or 5 discs on it. For enhanced essential oil absorption and bacterial growth suppression, plates were stored in the refrigerator for 30 minutes. The plates were inverted and incubated for 24 hours at 37ºC. After incubation, antibacterial activity was determined by measuring the zone of inhibition (ZOI) in millimeters (including the 6mm disc).

The test was repeated three times to acquire the confirmed result. The standard antibiotic in this trial was azithromycin (15g/disc), manufactured by NISSUI Pharmaceuticals Co.LTD (Japan).

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration of oils (MIC) was defined as the lowest concentration of oils that prohibited bacteria from growing on a plate. The CAR and CN MICs were determined using the disc diffusion technique on Mueller-Hinton agar plates. MBC was determined using a broth microdilution test and was defined as the smallest amount of oils required to have bactericidal action. The CAR and CN concentrations ranged from 1.25% to 0.078% (v/v).

Evaluation of the synergistic action of Carvacrol and Cinnamaldehyde with Nisin

The disc diffusion method was used to determined the synergistic antimicrobial potential of CAR and CN with nisin (SIGMA-ALDRICH). A very minute concentration (0.16%) of the antimicrobials was used to impregnate the filter paper disc. The discs contained nisin, CAR, and CN alone or combined the essential oils with nisin (0.16% CAR/CN+0.16% Nisin). The combined antimicrobial activity of CAR/CN and nisin against the test organisms were compared with the activity of individual one. The test was performed under similar environmental conditions stated previously.

Statistical analysis

Each experiment's ZOIs were determined as mean SD using triplicated data. The student's t-test was used to determine the relevance of diverse data in the Microsoft Excel application. Significant differences in the data were determined using the most negligible significant differences at the 5% significance level.

Results

Dose-dependent antimicrobial activity of Carvacrol and Cinnamaldehyde

The antimicrobial activity of CAR and CN was found to be dose-dependent which the increasing ZOI indicated in correspondence with higher CAR/CN concentrations. Both CAR and CN showed potential antimicrobial activity against the test organisms at all three concentrations (2.5%, 5%, and 10% v/v). CAR showed the most antimicrobial activity against *K. pneumoniae* (26-62 mm) and *S. flexneri* (30-57 mm) at three concentrations. *B. pumilus* (21-47 mm) and *B. amyloliquefaciens* (20-41 mm) were also sufficiently inhibited. The inhibition zones achieved against other tested organisms were also remarkable. 10% CAR inhibited *K. pneumoniae* (62.25mm) the most and *E. coli* (34mm) the least. The highest inhibition zone by 5% CAR was observed for *K. pneumoniae* (55.11mm) and the lowest for *M. luteus* and *E. coli* (30mm). Antimicrobial activity of 2.5% CAR was 15.3mm-29.71mm lower than 10% CAR. The *P. aeruginos*a strain used in the current study resisted the effect of CAR at all three concentrations but showed sensitivity to CN. The inhibitory activity exerted by CN was also satisfactorily high. At 10% CN concentration, the ZOI for the selected organisms ranged from 19.3 to 41.5 mm. Maximum and minimum inhibition was exhibited by *B. cereus* (41.5 mm) and *P.* aeruginosa (19.3mm), respectively. The 5% CN showed ZOI ranging from 12.2 to 20 mm, maximum for *B. cereus* (20 mm) and minimum for *P. aeruginosa* (12.2 mm). At 2.5% concentration, CN presented 9 mm to 20 mm ZOI, inhibiting *B. pumilus* (20 mm) the most and *P.* aeruginosa (9.0 mm) the least. Comparative analysis revealed CAR to be more effective than CN against the tested strains except for *P. aeruginosa*. Results for screening the antimicrobial activity of CAR and CN at three different concentrations (2.5%, 5% and 10%) are summarized in Table 1.

Comparative analysis of the antimicrobial activity of CAR and CN with standard antibiotic

The diameters of ZOI obtained against CAR and CN were compared to those obtained against standard antibiotic azithromycin (15 µg/disc) using student's t test analysis (Figure 1 and Figure 2). At 5% and 10% CAR concentrations, all the test bacteria provided significantly higher antimicrobial activity than the standard antibiotic, where p-value was £ 0.001 (Figure 1). At 2.5% CAR concentration, *B.cereus, B. pumilis*, *B. amyloliquefaciens* provided ZOI significantly smaller than the standard antibiotic with p £ 0.005 for *B.cereus* and P £ 0.001 for the rest two. The difference of ZOI against *S. typhi, S. flexneri, V. cholerae* was non-significant in the case of AZM and 2.5% CAR. *K. pneumoniae, M. luteus* and *E. coli* provided ZOI significantly higher than the standard antibiotic, even at 2.5% CAR concentration, where p-value was £ 0.001 for the first two and £ 0.005 for the rest. *P. aeruginosa* was resistant to both the standard antibiotic and CAR but was sufficiently reduced by CN.

Table 1. *Antimicrobial activity of Carvacrol, Cinnamaldehyde and a standard antibiotic Azithromycin*

Organisms	Zone of inhibition (in mm)						
	Antimicrobial agents used						Standard antibiotic
	CAR (% v/v)			CN (% v/v)			AZM
	10	5	2.5	10	5	2.5	(15µg/disc)
B. cereus	45.50±0.58	38.40±0.55	17.60±0.42	41.5 ± 1.0	20.0 ± 0.5	19.0 ±0.1	20.33±0.58
B. pumilus	47.75±0.50	38.44±0.58	21.20±0.45	26.8 ± 0.5	16.1 ± 0.5	20.0 ±0.5	25.67±0.58
B. amyloliquefaciens	41.30±0.45	35.50±0.53	20.75±0.50	29.8 ± 0.5	15.8 ± 0.6	12.0± 0.1	27.88±0.63
M. luteus	44.88±0.25	30.33±0.58	18.67±0.58	ND	ND	ND	6.67±0.58
S. typhi	45.60±0.55	35.25±0.50	15.89±0.42	32.2 ± 1.0	14.7 ± 0.5	14.0± 0.1	14.67±0.58
S. flexneri	57.63±0.48	35.75±0.50	30.43±0.53	ND	ND	ND	30.17±0.29
E. coli	34.00±0.71	30.75±0.50	18.70±0.150	34.3 ± 1.5	16.4 ± 0.5	15.0 ±0.2	14.83±0.29
V. cholerae	40.60±0.55	35.17±0.41	18.13±0.25	37.0 ± 1.5	19.8 ± 0.6	13.5± 0.4	17.67±0.58
K. pneumoniae	62.25±0.88	55.11±0.78	26.14±0.69	28.2 ± 0.5	14.4 ± 0.5	13.0 ±0.1	14.17±0.29
P. aeruginosa	0	0	0	19.3 ± 0.5	12 2 ± 0.5	09.0 ±0.1	0

Mean (mm) ± Standard deviation, (n=3), p<0.05, CAR= Carvacrol, CN= Cinnamaldehyde, AZM=azithromycin, ND: Not Determined

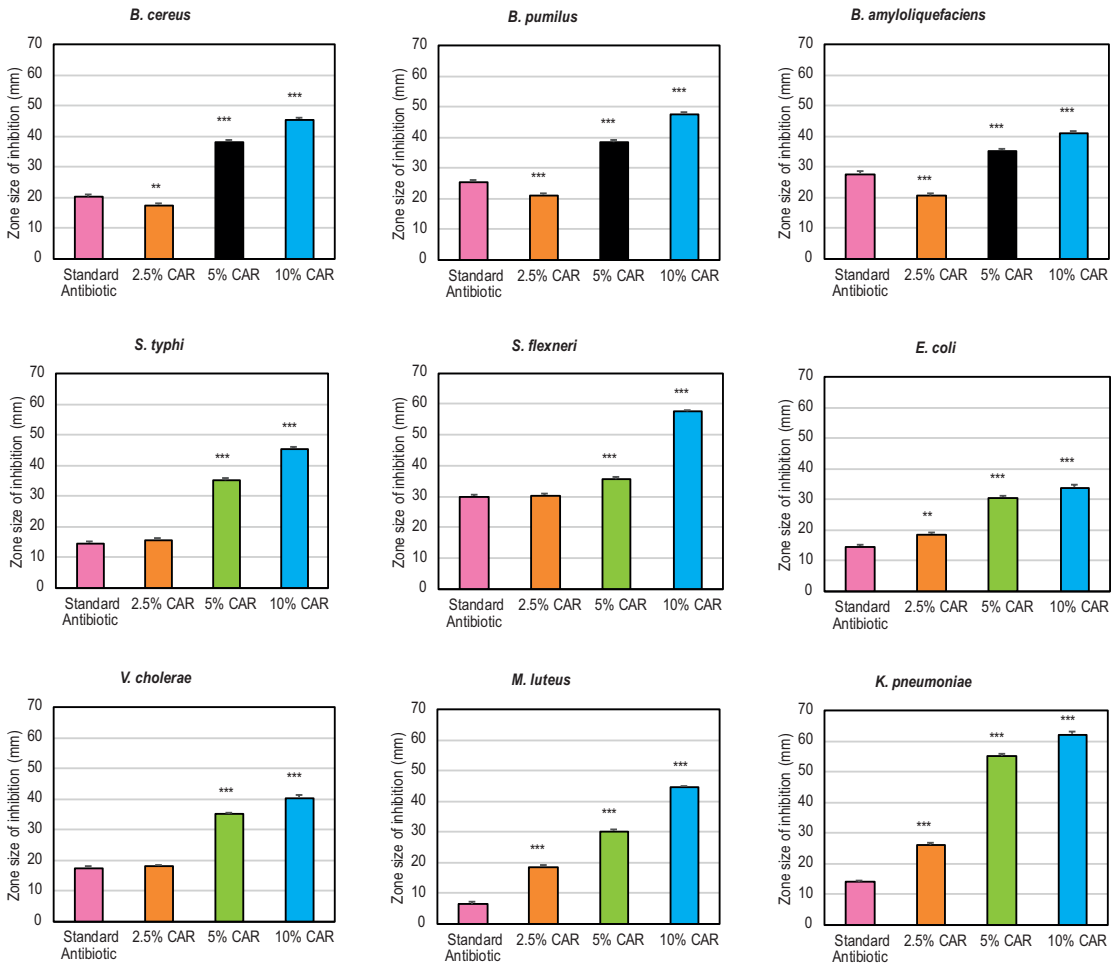

Fig. 1. *Graphical representation for the comparative analysis of the antimicrobial activity of Carvacrol (CAR) with standard antibiotic (Azithromycin, 15µg/disc) as a control. The x axis and Y axis respectively determine the zone of inhibition in mm and the antimicrobials used. P value was determined using student's t-test analysis with a significance of P£ 0.05 *, p £ 0.005 **, P £ 0.001 ***.*

Antimicrobial activity of 10% CN against *B. cereus, S. typhi, E. coli, V. cholerae, K. pneumoniae, P. aeruginosa* was significantly higher than the standard antibiotic with P £ 0.001 (Figure 2). *B. amyloliqufaciens* was also sufficiently inhibited by 10% CN rather than AZM, although the p-value was £ 0.05. Growth inhibition of *B. pumilus* by 10% CN and AZM showed statistically non-significant differences. Standard antibiotics showed significantly higher antimicrobial activity than 2.5% and 5% CN in the case of *B. pumilus and B. amyloliquefaciens* (P £ 0.001). Although the standard antibiotic exhibited higher antimicrobial activity than 2.5% and 5% CN, the statistical difference was non-significant except for *B. cereus* (P£ 0.05) and *V. cholerae* (P£ 0.005). Noteworthy, the inhibitory effect of 5% CN against *E. coli and* 2.5% and 5% CN against *P. aeruginosa* was significantly higher than the standard antibiotic (P£ 0.05 and P £ 0.001, respectively).

Minimum Inhibitory Concentration of Carvacrol and Cinnamaldehyde

MIC values of CAR against the test organisms ranged from 0.08% to 0.16%, with MBC values ranging from 0.16% to 0.31%. *B. pumilus* and *B. amyloliquefaciens* had the lowest CAR MIC values (0.08 percent). According to CN, the examined species had MIC values ranging from 0.16 percent to 0.31 percent. The MBC values for CN ranged from 0.31 to 0.63 percent. *S. typhi, E. coli*, and *V. cholerae* had the highest MBC values of CN. Figure 3 summarizes the findings of the MIC and MBC determinations.

Synergistic effect of Carvacrol and Cinnamaldehyde with Nisin:

The combinatorial antimicrobial effect of CAR and CN with Nisin was determined using low concentration of antimicrobials (0.16% v/v). At this low concentration, none of the antimicrobials could individually exert any remarkable antimicrobial activity. Interestingly, the combinatorial effect of the agents was satisfactorily high even at this minute concentration. CAR (0.16%) provided slightly higher antimicrobial activity than Nisin (0.16%) or CN (0.16%), although the difference did not reflect any statistically significant value. Both CAR (0.16%) + Nisin (0.16%) and CN (0.16%) + Nisin (0.16%) showed strong synergism in the case of all the test organisms, which was validated by the statistical difference among ZOIs in comparison with nisin alone. The highest

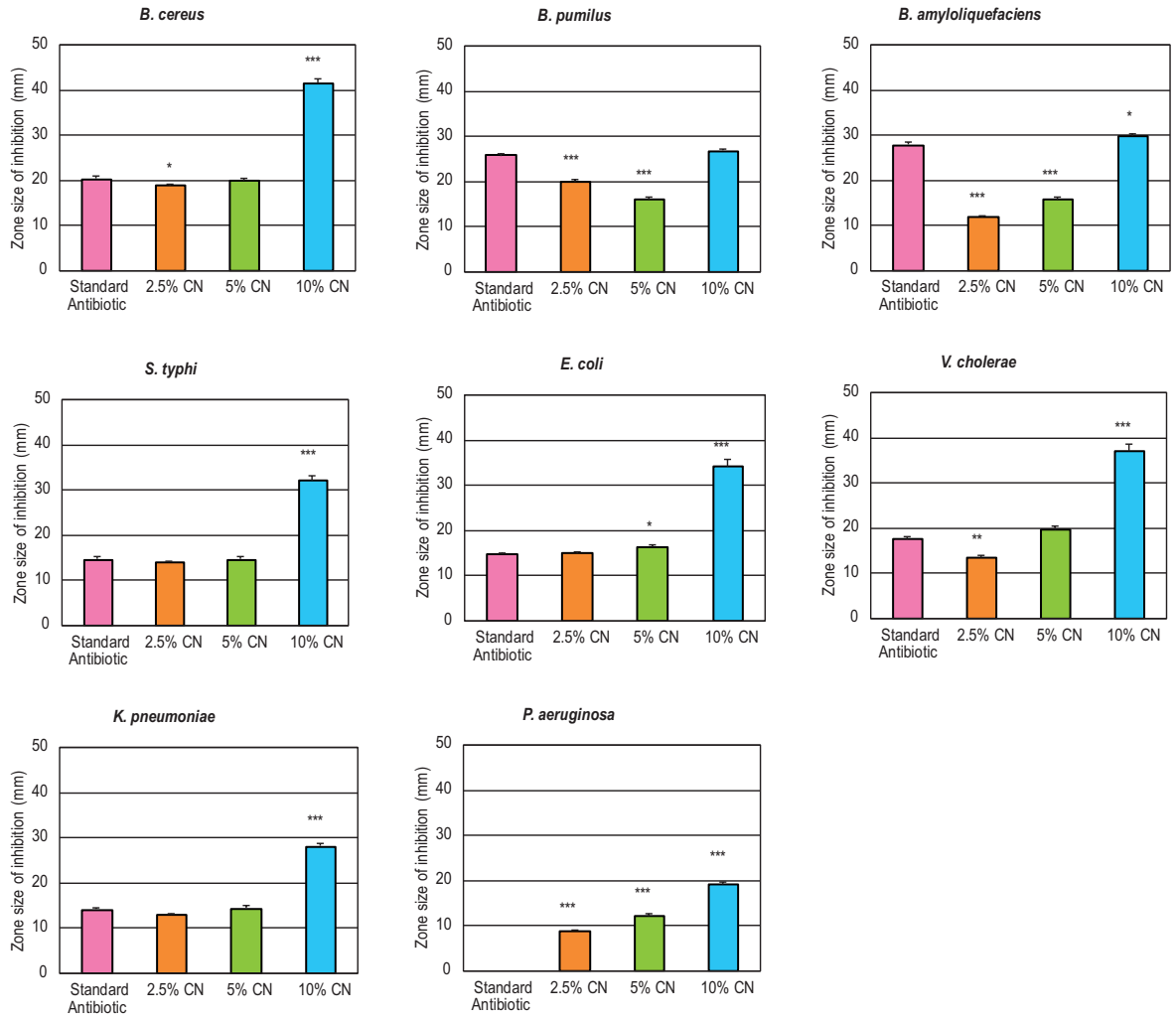

Fig. 2. *Graphical representation for the comparative analysis of the antimicrobial activity of Cinnamaldehyde (CN) with standard antibiotic (Azithromycin, 15µg/disc) as a control. The x axis and Y axis respectively determine the zone of inhibition in mm and the antimicrobials used. P value was determined using student's t-test analysis with a significance of P£ 0.05 *, p £ 0.005 **, P £ 0.001 ***.*

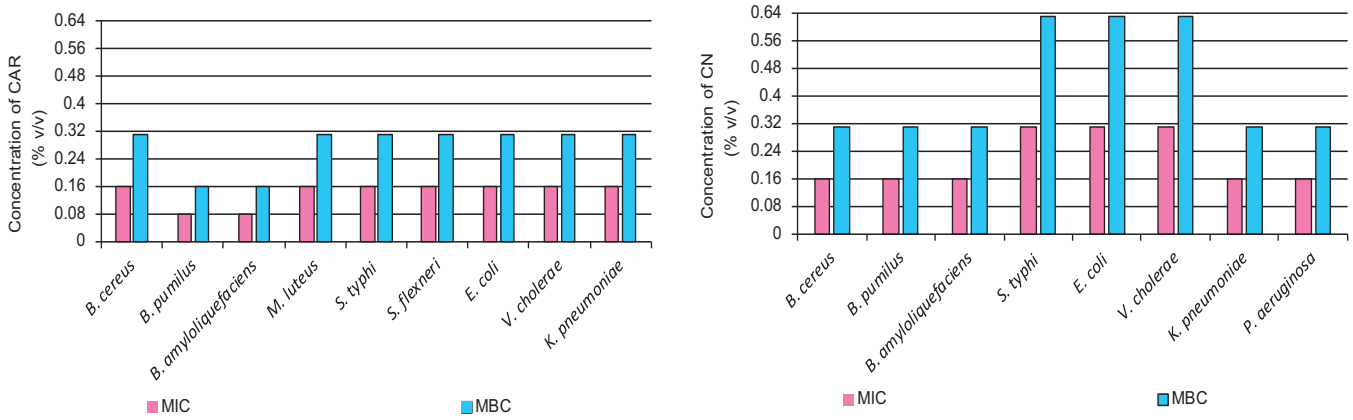

Fig. 3. *MIC and MBC values of Carvacrol (A) and Cinnamaldehyde (B) against the test organisms. X axis shows the concentration (% v/v) of antimicrobials used and Y axis shows the test organisms of concern. MIC values are represented in grey colour while MBC in black.*

synergism was observed for *B. amyloliquefaciens* challenged with CAR + Nisin (15.17mm ZOI). *S. flexneri, K. pneumoniae and B. cereus were* also sufficiently inhibited by CAR+Nisin. ZOI for the synergistic effect of CAR with Nisin ranged from 8.33mm to 15.17mm. However, the resistant *P. aeruginosa* strain was still able to overcome the combinatorial effect of CAR+Nisin, showing no growth inhibition. A combination of CN with Nisin also displayed higher antimicrobial activity than Nisin or CN alone, which was reflected by its significantly larger ZOI (9.0 mm to 14.3 mm, P £ 0.001) than nisin alone. The results of synergistic effects are provided in Figure 4 and Table 2.

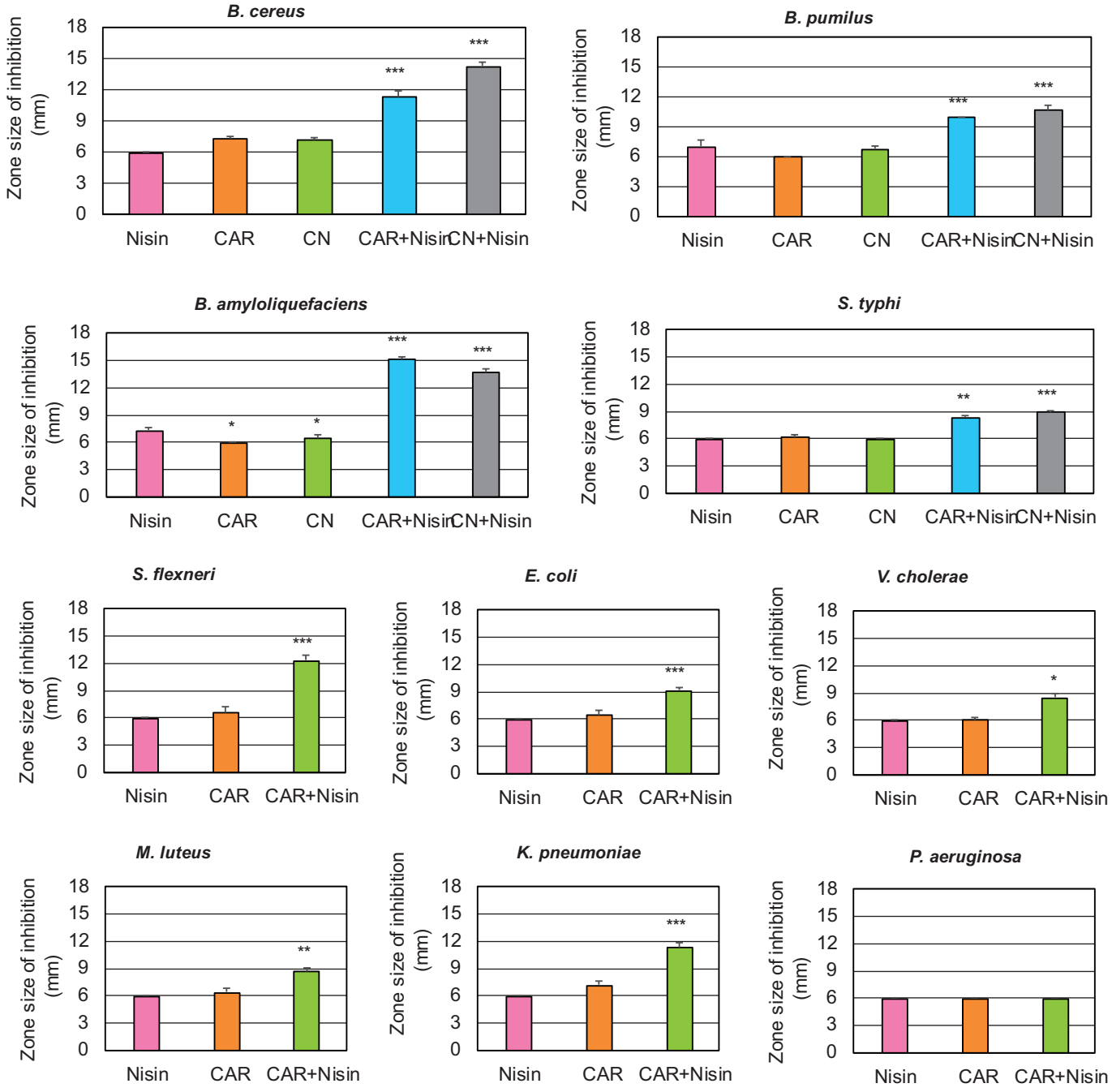

Fig. 4. *Graphical representation for the individual and combinatorial antimicrobial activity of Carvacrol (CAR) and Cinnamaldehyde (CN) with Nisin. Sensitivity patterns of various microbial strains against Nisin (0.16%), CAR (0.16%), CN (0.16%), CAR (0.16%) + Nisin (0.16%) and CN (0.16%) + Nisin (0.16%) are shown. The x axis and Y axis respectively determine the zone of inhibition in mm and the antimicrobials used. A zone diameter of 6mm (disc size) indicate no growth inhibition. Student's t-test analysis was used to determine the significance of difference among various treatments using nisin (0.16%) as a control where P £ 0.05 *, p £ 0.005 ** and P £ 0.001 ***.*

Discussion

Because of its helpful and safe reputation, the search for natural antimicrobial chemicals has intensified. Plant essential oils are rich in novel antibacterial chemicals with a green label, potent against microorganisms that cause food poisoning. Their use in food preservation, medicines, and natural remedies has been around for a long time[19, 20].

The influence of two key plant essential oil constituents (carvacrol and cinnamaldehyde) on the growth of foodborne pathogens and spoilage bacteria is described in this study. CAR and CN showed satisfactory inhibitory activity towards both Gram-positive and Gram-negative bacteria, suggesting their broad-spectrum applicability. While several experts suggest that carvacrol exerts more significant antimicrobial activity against Gram-positive than Gram-negative bacteria[21], the hypothesis is not out of contradiction. Carvacrol has been reported as a potent growth inhibitor for the Gram-negative bacilli with antimicrobial effects ranging from 62-250 Î¼g/mL[22]. Shen et al. reported that both Gram-positive and Gram-negative bacterial cell membrane integrity is profoundly damaged due to exposure to MIC level of CN[23].

Our study does not report any remarkable difference between the CAR and CN sensitivity of Gram-positive and Gram-negative bacteria, thus supporting the notion that the agents are equally applicable to combat a broad spectrum of foodborne pathogens. The bactericidal activity of CAR and CN was found to be dose dependent, with satisfactory antimicrobial activity even at 2.5% (v/v) concentration and gradually increasing due to increased concentration. A study carried out to evaluate the antimicrobial effect of 96 essential oils and 23 oil compounds also corroborates to the current study concluding that the bactericidal action of CAR and CN is dose-related. The researchers also found carvacrol as one of the major antimicrobials active against *E. coli, S. enterica* and *L. monocytogenes*[24]. *Klebsiella pneumoniae* and *Shigella flexneri* exhibited highest sensitivity to CAR at 2.5% to 10% concentrations which provides an indication that diseases caused by *Klebsiella pneumoniae* and *Shigella flexneri* (especially diarrhea and shigellosis) can be removed to a great extent by using CAR in food. Gram-positive *Bacillus pumilus* and *Bacillus amyloliquefaciens* were also significantly inhibited. The inhibition zones against other tested organisms were also remarkable (Table 1). Gram-positive *B. cereus* was inhibited mainly by CN, even though the fact does not flow any glad tidings toward the gram-negative ones. CN showed a potential inhibitory effect against azithromycin-resistant *P. aeruginosa* strain. This may lie in the mode of actions of CN, which mainly acts on bacterial cell membrane[25] while azithromycin needs to cross the membrane barrier to act as a protein synthesis inhibitor.

The continual rise in the emergence of antibiotic resistance superbugs urges us to find alternative solutions; CAR and CN can emancipate humankind from this curse. Student's t-test analysis revealed 10% CAR and CN to be significantly more effective than the standard antibiotic in the case of all the tested organisms (Figure 1 and Figure 2). MIC values of CAR (range: 0.08% to 0.31%) and CN (range: 0.16% to 0.31%) were very low, indicating both the agent to be suitable for selection against foodborne pathogens. The previous investigation reported that Shiga -like toxin gene stx2 in EHEC is downregulated by CN at a concentration of 0.01% v/v[26], which is even below its MIC level.

The intense flavor of CAR and CN may affect the organoleptic properties of foods due to their application in high concentrations, which is a significant reason behind their limited application as a food preservative. Combining these agents with other natural preservatives may offer desired antibacterial effect at a lower concentration, thus retaining the taste, flavor, and aroma of food commodities[27]. For using CAR and CN as food additives, a study was performed to determine the synergistic effect of these agents with nisin. A low concentration of these antimicrobials (0.16%) was used to determine the synergistic effect. At this very little concentration, the synergistic effect was found up to the mark, though the individual antimicrobials could not provide any unusual activity (Figure 4). This study provides insight into how the minute concentration of CAR+Nisin or CN+Nisin can be used as an excellent antimicrobial agent against foodborne pathogens, and thus these combinations can be considered in the preparation of natural food preservatives. Synergism of CAR and CN with Nisin has been reported in several previous studies also[28-31] . The weak and robust synergy of CN+Nisin was observed for *B. cereus* and *S. typhi,* respectively. These synergisms can also be employed in combating bacterial drug resistance.

Overall, the study adds light to the continued understanding of the antimicrobial effects of natural compounds-carvacrol and cinnamaldehyde. Antimicrobial solid activity, lower minimum concentration for bactericidal action, and significant synergism of these agents with nisin indicate that CAR and CN can be used as an alternative to conventional antibiotics and their potential application as a food preservative. But making any conclusion about the efficiency of these agents requires further research and analysis, including safety investigations and in vivo studies.

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