Original article

Synergistic antibacterial activity of carvacrol loaded chitosan nanoparticles with Topoisomerase inhibitors and genotoxicity evaluation

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A B S T R A C T

The increasing prevalence of antibiotic resistant bacteria is a significant healthcare crisis with substantial socioeconomic impact on global community. The development of new antibiotics is both costly and time-consuming prompting the exploration of alternative solutions such as nanotechnology which represents opportunities for targeted drug delivery and reduced MIC. However, concerns have arisen regarding genotoxic effects of nanoparticles on human health necessitating an evaluation of nanoparticle induced DNA damage.

This study aimed to investigate the antibacterial potential of already prepared, characterized chitosan nanoparticles loaded with carvacrol and their potential synergism with Topoisomerase II inhibitors against *S. aureus*, *E. coli* and *S. typhi* using agar well diffusion, microdilution and checkerboard method. Genotoxicity was assessed through comet assay.

Results showed that both alone and drug combinations of varying concentrations exhibited greater zones of inhibition at higher concentrations. Carvacrol nanoparticles combined with ciprofloxacin and doxorubicin significantly reduced MIC compared to the drugs used alone. The MIC_{50} values for ciprofloxacin were 35.8 μg/ml, 48.74 μg/ml, 35.57 μg/ml while doxorubicin showed MIC_{50} values of 20.79 μg/ml, 34.35 μg/ml, 25.32 μg/ml against *S. aureus*, *E. coli* and *S. typhi* respectively. The FICI of ciprofloxacin and doxorubicin with carvacrol nanoparticles found ≤ 0.5 Such as 0.44, 0.44,0.48 for ciprofloxacin and 0.45, 0.45, 0.46 for doxorubicin against *S. aureus*, *E. coli* and *S. typhi* respectively revealed the synergistic effect. The analysis of comet assay output images showed alteration of DNA at high concentrations.

Our results suggested that carvacrol nanoparticles in combination with Topoisomerase inhibitors may prevent and control the emergence of resistant bacteria with reduced dose.

1. Introduction

Emergence of bacterial resistance persist as critical challenge in public health as it causes morbidity and mortality globally. According to WHO infectious diseases are second cause of death around the world. It is estimated that annually 2 million people suffer from infections with antibacterial resistance and by the year of 2050 the global mortality rate will reach to 10 million (Nair et al., 2022). Antibiotics revolutionized modern medicine for treating bacterial infections. However, misuse of antibiotics over the time led to rapid emergence of pathogenic strains with antibacterial resistance (Chakraborty et al., 2022). Ciprofloxacin is a fluoroquinolone Topoisomerase inhibitor that penetrates through bacterial membranes, targets the nucleus, inhibits replication, transcription and translation of DNA. Ciprofloxacin conventional dosage forms do not transport the drug at targeted site and efficacy has been reduced. Resistance mechanisms are also developed by some pathogenic bacteria such as *Salmonella typhi, Escherichia coli* and *Staphylococcus aureus* due to its extensive use (Foudraine et al., 2022). Amino acid substitution of quinolone resistance

determining region of DNA gyrase A and point mutation in DNA gyrase B causes resistance of *Salmonella typhi* to ciprofloxacin. In addition, mutations in Topoisomerase IV domains that are encoding it resulted in resistance of *Salmonella typhi* and elevated MIC of ciprofloxacin (Accou-Demartin et al., 2011). *Staphylococcus aureus* and *Escherichia coli* as the result of gene mutations possess ability to acquire resistance to ciprofloxacin (Guan et al., 2013). Doxorubicin is chemotherapeutic drug that also inhibits Topoisomerase enzymes. Previously described studies illustrated remarkable antibacterial activity of doxorubicin so it can present a viable option for repurposing (WC Soo et al., 2017).

Bacterial resistance emphasized to identify novel effective drug delivery and targeting approach. In this context, nanoparticles (NPs) as novel technology acted as indispensable viable therapeutic option with improved bioavailability, enhanced absorption and faster passage of drug into the cell (Bekele and Alamnie 2022). NPs offer opportunities to access antibacterial modalities novel to bacteria that do not fall in their natural defense arsenal. The therapeutic effect of nanomaterials is largely derived from nanoscale confinement of materials compounded with multivalent interactions and high surface to volume ratio (Munir and Ahmad 2022). NPs can penetrate bacterial cell membrane and interfere with molecular pathways. They signified synergy in combination with optimal antibiotics by enhancing the inhibitory effects of antibiotics and may assist in limiting the global crisis of emerging bacterial resistance (Lee et al., 2019).

Carvacrol is monoterpenoid found in oregano, marjoram and thyme that alters the permeability of bacteria and increases the uptake of antibiotics (Shakeri et al., 2019). Combination therapy using plant-based nanoparticles and Topoisomerase inhibitors may constitute promising candidate that will provide synergism with reduced MIC of antibiotics, reduced toxicity and enhanced spectrum of activity against resistant bacteria (Bazzaz et al., 2019). Genotoxicity of NPs assessed by single cell gel electrophoresis, where DNA damage is measured in single cells. By the exposure of electrophoresis breaks in DNA strands allow it to move out of the nucleoid that appeared as comet (Cordelli et al., 2021).

The aim of present study was evaluation of antibacterial activity of already prepared carvacrol loaded chitosan nanoparticles in combination with Topoisomerase inhibitors and *in vitro* assessment of genotoxicity.

2. Materials and methods

2.1. Collection of nanoparticles

The carvacrol loaded chitosan nanoparticles were manufactured through ionic gelation method during first component of the project. For this purpose, commercially available carvacrol (Sigma Aldrich) was mixed with different concentrations of commercially available chitosan (Sigma Aldrich) to attain different carvacrol to chitosan ratios such as 1:0, 1:0.25, 1:0.50, 1:0.75, 1:1 and 1:1.25. Based upon maximum yield, carvacrol to chitosan ratio 1:1 found suitable (results not shown here) and recommended for further studies. The vials containing carvacrol loaded chitosan nanoparticles in lyophilized form were collected from Quality Operations Laboratory, UVAS and characterized as per standard protocol.

2.2. Assessment of antibacterial activity

2.2.1. Collection of bacterial cultures

The activated and biochemically characterized culture of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* were collected from the Institute of Microbiology, UVAS and used as representative bacteria for evaluating the anti-bacterial potential of carvacrol, chitosan, ciprofloxacin, doxorubicin, carvacrol loaded chitosan nanoparticles and their combinations such as carvacrol-chitosan, carvacrol-ciprofloxacin, carvacrol-doxorubicin, carvacrol nanoparticles-ciprofloxacin, carvacrol nanoparticles-doxorubicin (1:1). The experiments were performed twice against each bacterium to record the results.

2.2.2. Antimicrobial susceptibility testing by disc diffusion method

In vitro assessment of resistance of each type of bacterium to ciprofloxacin or susceptibility to standard antibacterial agents was carried out through Kirby-Bauer disc diffusion method. Initially, each type of tested bacterium were grown in freshly prepared nutrient broth to attain 0.5 McFarland turbidity (1.5×10^8 CFU/ml) followed by swabbing on surface of Mueller Hinton agar in petri plates separately. Standard antibiotic discs such as ciprofloxacin (5 µg), Amikacin (30 µg), Doxycycline (30 µg), Gentamicin (10 µg), Tigecycline (15 µg), Ampicillin (10 µg), Trimethoprim/sulfamethoxazole (25 µg) and Polymyxin B (300 µg) were placed over the inoculated plates and they were incubated at 37 °C for 24 h. The diameter of zone of inhibition around the disc was measured with vernier caliper as suggested by CLSI guidelines (Maharjan et al., 2021).

2.2.3. Evaluation of antibacterial activity by agar well diffusion method

In vitro antibacterial activity of alone drugs such as carvacrol, chitosan, carvacrol NP, ciprofloxacin, doxorubicin and their combinations carvacrol-chitosan, carvacrol-ciprofloxacin, carvacrol-doxorubicin, carvacrol NPs-doxorubicin, carvacrol NPs-ciprofloxacin prepared in 1:1 ratio was evaluated by using agar well diffusion method. Initially two-fold serial dilutions of each drug and their combination were prepared in Mueller Hinton broth medium to attain varying concentration from 1000 to 1.95 µg/ml. In parallel, each type of tested bacterial culture was grown in nutrient broth to attain 1.5×10^8 CFU/ml and then swabbed on surface of Mueller Hinton agar on petri plates separately. Sterile borer was used to make wells (8 mm) on agar surface and sealed the well with molten agar. Subsequently, sample amounting to 100 µl from each dilution of above tested drugs were collected and poured in respective wells. The petri plates were incubated at 37 °C for 24 h and measured the zone diameter (mm) around the well (Chavez-Esquivel et al., 2021).

To determine susceptibility levels of ciprofloxacin against *S. aureus, E. coli* and *S. typhi,* the CLSI has set certain criteria. Specifically, for *S. aureus* a zone of inhibition greater than or equal to 21 mm indicates sensitivity, while measurements ranging from 16 to 20 mm represent intermediate levels and diameter less than or equal to 15 mm suggests resistance. As for *E. coli,* ciprofloxacin is considered sensitive if its zone of inhibition measures greater than or equal to 26 mm, intermediate if it falls between 22 and 25 mm and resistant if it is less than or equal to 21 mm. For *S. typhi* ciprofloxacin is regarded as sensitive if the zone of inhibition measures greater than or equal to 31 mm, intermediate if falls between 21 and 30 mm and below or equal to 20 mm deemed resistant. For carvacrol, chitosan, carvacrol NPs, doxorubicin and drug combinations zone of inhibition against tested bacterium is classified as sensitive if it measures greater than or equal to 20 mm, intermediate if it falls between 15 and 19 mm and resistant if it is less than or equal to 14 mm.

2.2.4. Determination of minimum inhibitory concentration by microdilution method

The minimum inhibitory concentration (MIC) of above said tested drugs and combinations was determined through microdilution method. Initially serially two-fold dilutions of each drug and combinations were prepared in respective wells of 96-well

micro-titration plate up to 10th well varying the concentration ranges from 1000 to 1.95 µg/ml keeping the 11th well as negative control. Simultaneously, 0.5McFarland standards of each representative bacterial suspensions were diluted 100x to attain density of 5×10^6 CFU/ml. Sample amounting to 50 µl from each diluted bacterial suspension were added in all wells of 96-well plate and then incubated at 37 °C for 24 h. Bacterial growth in each well was estimated through measuring absorbance at 600 nm by ELISA microplate reader. The minimum concentration of drugs at which 50% of bacterial growth was inhibited termed as MIC_{50} was calculated by using following formula (Alaqeel et al., 2021).

$$GrowthInhibition(\%) = \frac{MeanODoftestcontrol - MeanODofnegativecontrol}{MeanODofnegativecontrol} \times 100$$

2.2.5. Determination of synergistic potential by checkerboard method

The potential synergistic effect of carvacrol and carvacrol nanoparticles in binary combinations was evaluated through checkerboard assay. For this purpose, 100 µl of Muller Hinton broth was dispensed in all wells of 96-well micro-titration plate followed by addition of 100 µl of carvacrol, carvacrol nanoparticles in wells of 1st column and making two-fold serial dilutions up to 10th well of respective rows attaining the varying concentrations ranging from 1000 to 1.95 µg/ml respectively. Subsequently, 100 µl of chitosan, ciprofloxacin and doxorubicin were added in respective wells of 1st column and making two-fold serial dilutions vertically. Sample amounting to 50 µl (5×10^6 CFU/ml) from each bacterial suspension were added in all wells of 96-well plate and incubated at 37 °C for 24 h. Separated 96-well plates were reserved for each bacterial type. Fractional inhibitory concentration index (FICI) of drug combinations was determined by measuring optical density at 600 nm and following formula (Bellio et al., 2021).

$$FICI = FIC_A + FIC_B$$

Where, FIC_A = MIC of drug A in the combination/MIC of drug A alone; FIC_B = MIC of drug B in the combination/MIC of drug B alone.

The values of FICI ≤ 0.5, $> 0.5–1.0$, $> 1.0–4.0$, > 4.0 interpreted Synergistic, Additive, Indifferent (non-interactive), and Antagonistic effect (Sharma et al., 2020).

2.3. Genotoxicity evaluation by single cell gel electrophoresis (SCGE)/comet assay

Comet assay was employed for evaluation of genotoxic potential of carvacrol NPs, carvacrol-doxorubicin, carvacrol-ciprofloxacin, carvacrol NPs-doxorubicin and carvacrol NPs-ciprofloxacin. For this purpose, lymphocytes from sheep blood were separated by mixing with lymphocytes separating media in equal ratio in falcon tube. The blood suspension tube was centrifuged at 800x/45 min at room temperature and collected the buffy layer containing lymphocytes. The buffy layer was mixed with RPMI-1640 media in equal proportion and centrifuged at 800x/15 min followed by collection of lymphocyte pellet and counted through hemocytometer. Subsequently, serial two-fold dilutions of above said drugs with varying concentrations from 1000 to 31.25µg/ml were made and exposed each dilution to 100 µl of lymphocyte suspension respectively followed by incubation for 2 h. Following incubation 10 µl of treated lymphocytes cells were mixed with 65 µl of low melting agarose (LMA) spread on slides already prepared with normal melting agarose (NMA). The slides were kept on ice till hardening of agarose layer. The slides were treated with lysing solution and incubated in dark for 2-12hr. Following incubation, slides were treated with alkaline buffer and placed in electrophoresis tank. Electrophoresis was performed at 24 V, 300 mA for 30 min. Then, the slides were neutral-

ized using neutralizing buffer solution for 5 min and stained with ethidium bromide. The slides were examined at 250X under fluorescent microscope. Length and width was measured using comet score to quantify the DNA damage (Kousar and Javed 2015).

2.3.1. Comet scoring

Comet scoring was assigned to all slides by using J. Launcher image software as per concentration (1000–31.25 µg/ml) of above-mentioned drugs. DMSO (20%) and normal saline solutions were used as positive and negative control respectively. Undamaged cells were grouped under class 0, cells having tail length $\leq$ head diameter under class 1, Tail length > Head diameter; Tail length $\leq$ double the Head diameter under class 2 and Tail length > Head diameter under class 3. Damage index (DI) and genetic damage index (GDI) were calculated as following formula described by (Tutty et al., 2022).

Damage Index = Number of cells in Class 1 + (2 × Number of cells in Class 2) + (3 × Number of cells in Class 3).

Genetic Damage index GDI = Number of cells in Class 1 + (2 × No of the cells in Class 2) + (3 × No of cells in Class 3) / No of cells in Class 0 + No of cells in Class 1 + No of the cells in Class 2 + No of cells in Class 3.

3. Results

3.1. Antimicrobial susceptibility testing (AST) by disc diffusion method

Based upon Kirby-Bauer disc diffusion method, it was observed that representative bacteria such as *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* according to CLSI recommendations showed resistance towards ciprofloxacin and susceptibility towards standard antibiotic discs of Amikacin, Doxycycline, Gentamicin, Tigecycline, Ampicillin, Trimethoprim/sulfamethoxazole and Polymyxin B as shown in (Fig. 1).

3.2. Antibacterial effect evaluation by agar well diffusion method

The study assessed the antibacterial efficacy of various drugs and their combinations against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*. Results indicated that the diameter of the zone of inhibition increased proportionally with the dose of drugs for all tested bacteria. The drugs and their combinations dis-
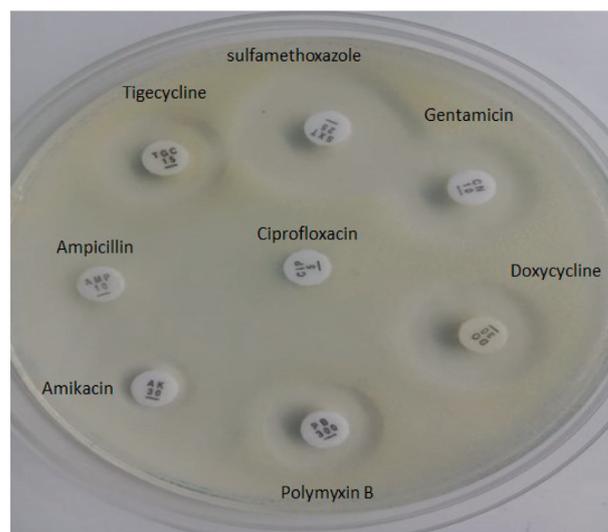
Fig. 1. Antimicrobial susceptibility test; Bacterial growth showed resistance towards Topoisomerase inhibitor ciprofloxacin.

played inhibition zones ranging from 1000 to 15.62 µg/ml, whereas other concentrations did not exhibit any inhibition zones. Notably, the combinations of drugs demonstrated significant antibacterial activity and resulted in larger zones of inhibition against the tested bacteria compared to the individual drugs.

The carvacrol nanoparticles exhibited a zone of inhibition measuring 22 mm each against *S. aureus, E. coli* and *S. typhi* at high concentration. However, when they were combined with ciprofloxacin, the zone of inhibition was significantly enhanced to 27 mm, 30 mm and 36 mm respectively, indicating that the combination demonstrated a stronger antibacterial effect as shown in Table 1.

3.3. Percentage inhibition and MIC$_{50}$ determination by micro dilution method

The micro-dilution method was used to determine the percentage inhibition and minimum inhibitory concentration (MIC) of various drugs and their combinations against *S. aureus*, *E. coli* and *S. typhi*. Percentage inhibition quantifies the extent to which the antibacterial agent restricts or prevents the growth of the target microbe. The results showed that carvacrol, chitosan, carvacrol NPs, ciprofloxacin, doxorubicin and their combinations had a significant inhibitory effect on the growth of these bacteria. At a concentration of 1000 µg/ml the maximum growth inhibitions for *S. aureus* were 86.47%-99.42% (Fig. 2A) while for *E. coli* and *S. typhi* they ranged from 70.68% to 98.63% (Fig. 2B) and 84.26%-97.26% (Fig. 2C) respectively. In contrast, at a concentration of 1.95 µg/ml the inhibition percentages were much lower for all treatments ranging from 0.38% to 7.93%. As the concentration of drugs and their combinations increased, the growth of tested bacteria decreased.

The MIC at 50% (MIC$_{50}$) refers to the concentration of an antibacterial agent that inhibits the growth of the microorganism by 50%. The MIC$_{50}$ value is useful for comparing the efficacy of different antibacterial agents. The study aimed to determine the minimum inhibitory concentration of drugs required to inhibit 50% of bacterial growth (MIC$_{50}$) and explore whether drug combinations could reduce the MIC$_{50}$ of individual drugs. The results showed that when carvacrol and chitosan were used alone their MIC$_{50}$ against *S. aureus* was 266.36 µg/ml and 72.04 µg/ml respectively. However, when combined, the MIC$_{50}$ decreased to 38.28 µg/ml. Similarly, ciprofloxacin and doxorubicin had MIC$_{50}$ values of 227.6 µg/ml and 72.48 µg/ml respectively against *S. aureus* when used alone. But when these drugs were combined with carvacrol, their MIC$_{50}$ values decreased to 99.28 µg/ml and 52.8 µg/ml respectively. Furthermore, the MIC$_{50}$ of carvacrol NPs against *S. aureus* was found to be 124.61 µg/ml but when combined with ciprofloxacin and doxorubicin its MIC$_{50}$ was drastically reduced to 35.8 µg/ml and 20.79 µg/ml respectively as shown in (Fig. 2A).

The effectiveness of carvacrol and chitosan against *E. coli* was observed at MIC$_{50}$ values of 355.5 µg/ml and 70.66 µg/ml respectively when used alone. However, when these two compounds

were combined the MIC$_{50}$ value decreased to 44.01 µg/ml. Similarly, ciprofloxacin and doxorubicin showed individual MIC$_{50}$ values of 175.15 µg/ml and 103.08 µg/ml against *E. coli* but when used in combination their MIC$_{50}$ values decreased to 97.47 µg/ml and 56.25 µg/ml respectively. Carvacrol NPs demonstrated a MIC$_{50}$ value of 303.15 µg/ml against *E. coli* which reduced considerably to 48.74 µg/ml and 34.35 µg/ml when combined with ciprofloxacin and doxorubicin respectively as shown in (Fig. 2B). Moving on to *S. typhi* the MIC$_{50}$ values of carvacrol and chitosan were observed at 284.5 µg/ml and 75.79 µg/ml respectively when used separately. When these compounds were combined their MIC$_{50}$ value dropped to 44.34 µg/ml. For ciprofloxacin and doxorubicin used individually against *S. typhi* their observed MIC$_{50}$ values were 197.48 µg/ml and 103.08 µg/ml respectively. But when used in combination they showed a reduction in their MIC$_{50}$ values to 105 µg/ml and 51.88 µg/ml respectively. Lastly, carvacrol NPs exhibited a MIC$_{50}$ value of 117.5 µg/ml against *S. typhi* which decreased remarkably to 35.57 µg/ml and 25.32 µg/ml when combined with ciprofloxacin and doxorubicin respectively as shown in (Fig. 2C).

In a study comparing the effectiveness of drugs and their combinations, it was found that combining carvacrol with chitosan, ciprofloxacin or doxorubicin reduced the MIC$_{50}$ value of each individual drug. However, when carvacrol nanoparticles were combined with ciprofloxacin and doxorubicin there was a significant reduction in MIC$_{50}$ against *S. aureus*, *E. coli* and *S. typhi* as compared to the nanoparticles used alone as shown in (Fig. 2D).

The statistical analysis of MIC$_{50}$ for drugs and their combinations against bacteria (*S. aureus, E. coli, S. typhi*) was performed using randomized complete block design (RCBD). In case of drugs and their combinations (p-value < 0.05) was considered to have a significant effect on MIC$_{50}$ values. Conversely (p-value > 0.05) for bacteria indicated non-significant effect on the MIC$_{50}$ values.

3.4. Evaluation of synergistic effect by checkerboard method

Interaction between drug combinations was determined by Fractional inhibitory concentration index (FICI). Among all the drug combinations carvacrol NPs with ciprofloxacin and doxorubicin exhibited synergistic effect (FICI ≤ 0.5) while rest of combinations showed additive effect (FICI > 0.5–1.0) against *S. aureus, E. coli,* and *S. typhi* as shown in Table 2.

3.5. Genotoxicity evaluation by single cell gel electrophoresis (SCGE)/comet assay

The genotoxicity of combinations of carvacrol and carvacrol NPs with ciprofloxacin and doxorubicin was investigated using comet assay at concentrations ranging from 1000 to 31.25 µg/ml. The results showed that carvacrol NPs caused genetic damage at high concentrations of 1000 µg/ml and 500 µg/ml with a genetic damage index (GDI) of 0.16 and 0.08 respectively. However, all other

Table 1
Zones of inhibition of alone and drug combinations against *S. aureus, (E. coli), and S. typhi.*

Dilutions (µg/ml)	CAR	CHITO	CAR.NPs	CIP	DOXO	CAR + CHITO	CAR + CIP	CAR + DOXO	CAR.NPs + CIP	CAR. NPs + DOXO
1000	19(20)20	15(17)17	22(22)22	14(12)13	18(17)17	19(19)19	24(28)32	20(20)21	27(30)36	22(22)23
500	17(18)18	13(15)15	20(21)21	12(10)11	16(16)16	15(17)17	22(26)28	18(19)19	24(27)34	20(20)21
250	15(16)16	11(12)12	18(19)19	10(8)10	15(15)15	12(15)15	20(24)25	15(18)17	21(25)31	18(18)19
125	12(14)14	0(0)0	0(0)0	8(0)8	0(0)0	10(11)11	18(21)22	12(15)15	19(22)27	15(15)15
62.5	10(12)12	0(0)0	0(0)0	0(0)0	0(0)0	8(8)8	15(18)20	10(12)12	16(20)25	12(10)12
31.25	8(8)8	0(0)0	0(0)0	0(0)0	0(0)0	0(0)0	11(15)18	0(0)0	11(18)22	0(0)0
15.62	0(0)0	0(0)0	0(0)0	0(0)0	0(0)0	0(0)0	0(0)0	0(0)0	8(15)10	0(0)0

CAR: Carvacrol; CHITO: Chitosan; CIP: Ciprofloxacin; DOXO: Doxorubicin; NPs: Nanoparticles.
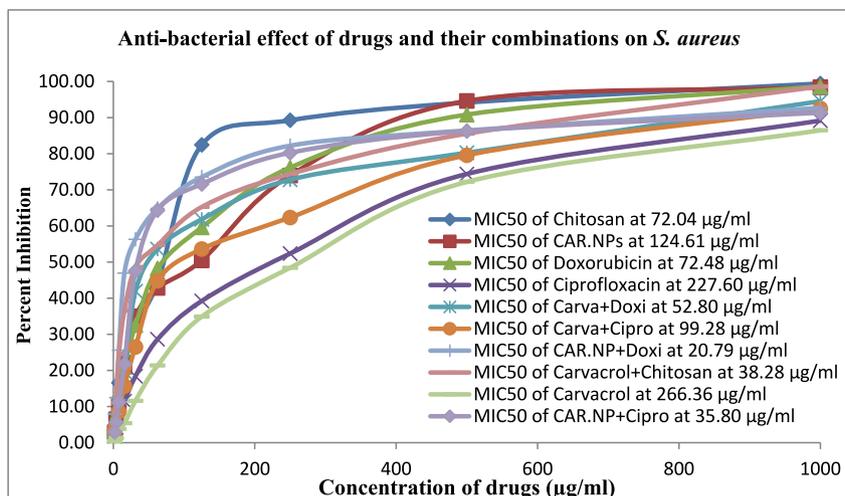
Fig. 2A. Percentage inhibition and MIC$_{50}$ of drugs and their combinations on *S. aureus* (A); MIC$_{50}$: concentration of drug (µg/ml) to inhibit 50% growth of *S. aureus*. MIC$_{50}$ was lowered from 266.36 to 124.61 µg/ml for carvacrol alone and carvacrol NPs respectively; MIC$_{50}$ was lowered from 227.60 to 99.28 and 35.80 µg/ml for ciprofloxacin alone and combination with carvacrol and carvacrol NPs respectively; MIC$_{50}$ for doxorubicin alone found 72.48 and 52.80, 20.79 µg/ml in combination with carvacrol and carvacrol NPs respectively; MIC$_{50}$ for chitosan found 72.04 and lowered to 38.28 µg/ml in combination with carvacrol.
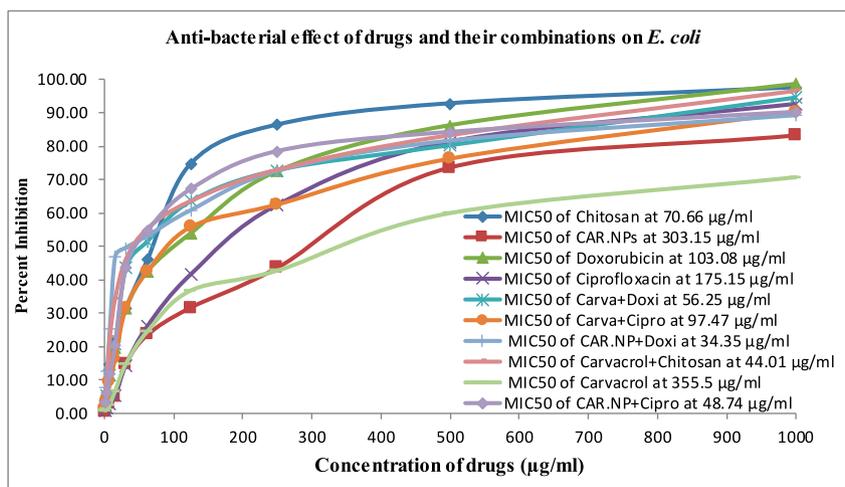
Fig. 2B. Percentage inhibition and MIC$_{50}$ of drugs and their combinations on *E. coli* (B); MIC$_{50}$: concentration of drug (µg/ml) to inhibit 50% growth of *E. coli*. MIC$_{50}$ was lowered from 355.5 to 303.15 µg/ml for carvacrol alone and carvacrol NPs respectively; MIC$_{50}$ was lowered from 175.15 to 97.47 and 48.74 µg/ml for ciprofloxacin alone and combination with carvacrol and carvacrol NPs respectively; MIC$_{50}$ for doxorubicin alone found 103.08 and 56.25, 34.35 µg/ml in combination with carvacrol and carvacrol NPs respectively; MIC$_{50}$ for chitosan found 70.66 and lowered to 44.01 µg/ml in combination with carvacrol.

concentrations tested were found to be safe. The mean tail length of DNA in cells exposed to carvacrol NPs at the high concentrations of 1000 µg/ml, 500 µg/ml was 0.29 ± 0.32 µm and 0.24 ± 0.34 µm respectively which was much lower than the positive control (20% DMSO) where the tail length was 13.2 ± 0.49 µm.

The combination of carvacrol-doxorubicin had a GDI of 0.2 and 0.12 at high concentrations of 1000 µg/ml and 500 µg/ml respectively while rest of concentrations were safe. The mean tail length of DNA in cells exposed to this combination was 0.32 ± 0.32 µm and 0.18 ± 0.27 µm respectively which was less damaged compared to the positive control. The Carvacrol NPs-doxorubicin combination exhibited had a GDI of 0.28 at high concentration 1000 µg/ml while rest of concentrations were found to be safe. The mean tail length of DNA by exposure of this combination was 0.22 ± 0.32 µm which was less damaged compared to positive control.

Similarly, the combination of carvacrol-ciprofloxacin exhibited a GDI of 0.12 and 0.04 at high concentrations 1000 µg/ml and

500 µg/ml respectively while remaining concentrations were safe. The mean tail length of DNA in cells exposed to this combination was 0.26 ± 0.32 µm and 0.21 ± 0.27 µm respectively which was less damaged compared to the positive control. The carvacrol NPs-ciprofloxacin combination had a GDI of 0.24 at high concentration 1000 µg/ml while rest of concentrations were safe. The mean tail length of DNA by exposure of this combination was 0.198 ± 0.32 µm which was less damaged compared to the positive control. As the concentration of drug increased there was an observed increase in GDI. Carvacrol NPs and drug combinations at their respective concentrations showed Class I DNA damage as shown in (Fig. 3).

The statistical analysis of genetic damage index of drugs and their combinations in relation to dilutions was conducted using RCBD. In case of drugs and their combinations (p-value > 0.05) indicated non-significant effect on genetic damage index. However, the p-value of dilutions < 0.05 was obtained which interpreted the significant effect on genetic damage index.
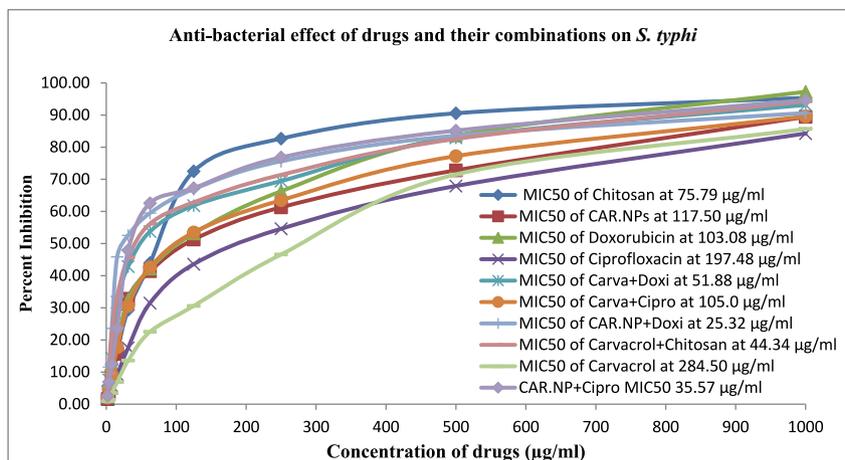
Fig. 2C. Percentage inhibition and MIC$_{50}$ of drugs and their combinations on *S. typhi* (C); MIC$_{50}$: concentration of drug (μg/ml) to inhibit 50% growth of *S. typhi*. MIC$_{50}$ was lowered from 284.50 to 117.50 μg/ml for carvacrol alone and carvacrol NPs respectively; MIC$_{50}$ was lowered from 197.48 to 105.0 and 35.57 μg/ml for ciprofloxacin alone and combination with carvacrol and carvacrol NPs respectively; MIC$_{50}$ for doxorubicin alone found 103.08 and 51.88, 25.32 μg/ml in combination with carvacrol and carvacrol NPs respectively; MIC$_{50}$ for chitosan found 75.79 and lowered to 44.34 μg/ml in combination with carvacrol.
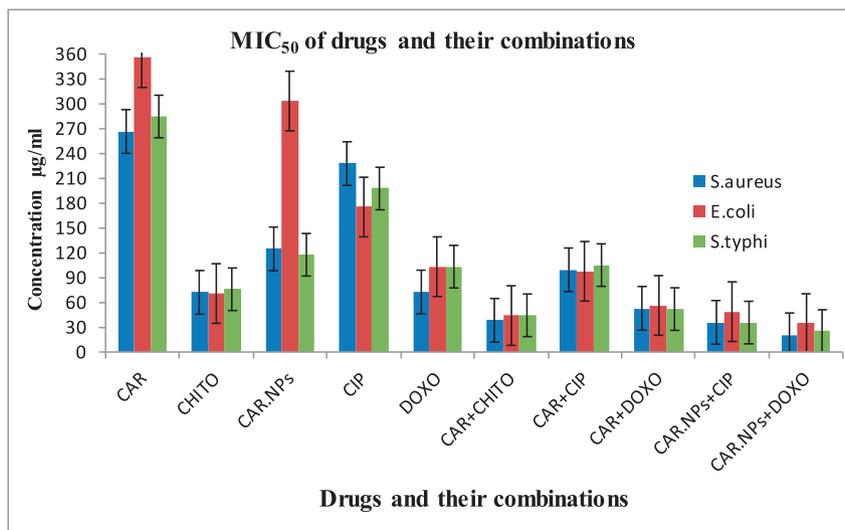
Fig. 2D. Comparative effect of drugs and their combinations on MIC$_{50}$ against different bacteria (D); CAR: carvacrol; CHITO: chitosan; CAR.NPs: carvacrol nanoparticles; CIP: ciprofloxacin; DOXO: doxorubicin.

Table 2
FICI of drug combinations against *S. aureus/E. coli/S. typhi*.

Compounds	Bacteria	FIC-A	FIC-B	FICI	Outcomes
Carvacrol-Chitosan	*S. aureus*	0.14	0.53	0.68	Additive
	E. coli	0.12	0.62	0.75	Additive
	S. typhi	0.16	0.59	0.74	Additive
Carvacrol- Ciprofloxacin	*S. aureus*	0.37	0.44	0.81	Additive
	E. coli	0.27	0.56	0.83	Additive
	S. typhi	0.37	0.53	0.90	Additive
Carvacrol- Doxorubicin	*S. aureus*	0.20	0.73	0.93	Additive
	E. coli	0.16	0.55	0.70	Additive
	S. typhi	0.18	0.50	0.69	Additive
Carvacrol NPs- Ciprofloxacin	*S. aureus*	0.29	0.16	0.44	Synergistic
	E. coli	0.16	0.28	0.44	Synergistic
	S. typhi	0.30	0.18	0.48	Synergistic
Carvacrol NPs- Doxorubicin	*S. aureus*	0.17	0.29	0.45	Synergistic
	E. coli	0.11	0.33	0.45	Synergistic
	S. typhi	0.22	0.25	0.46	Synergistic
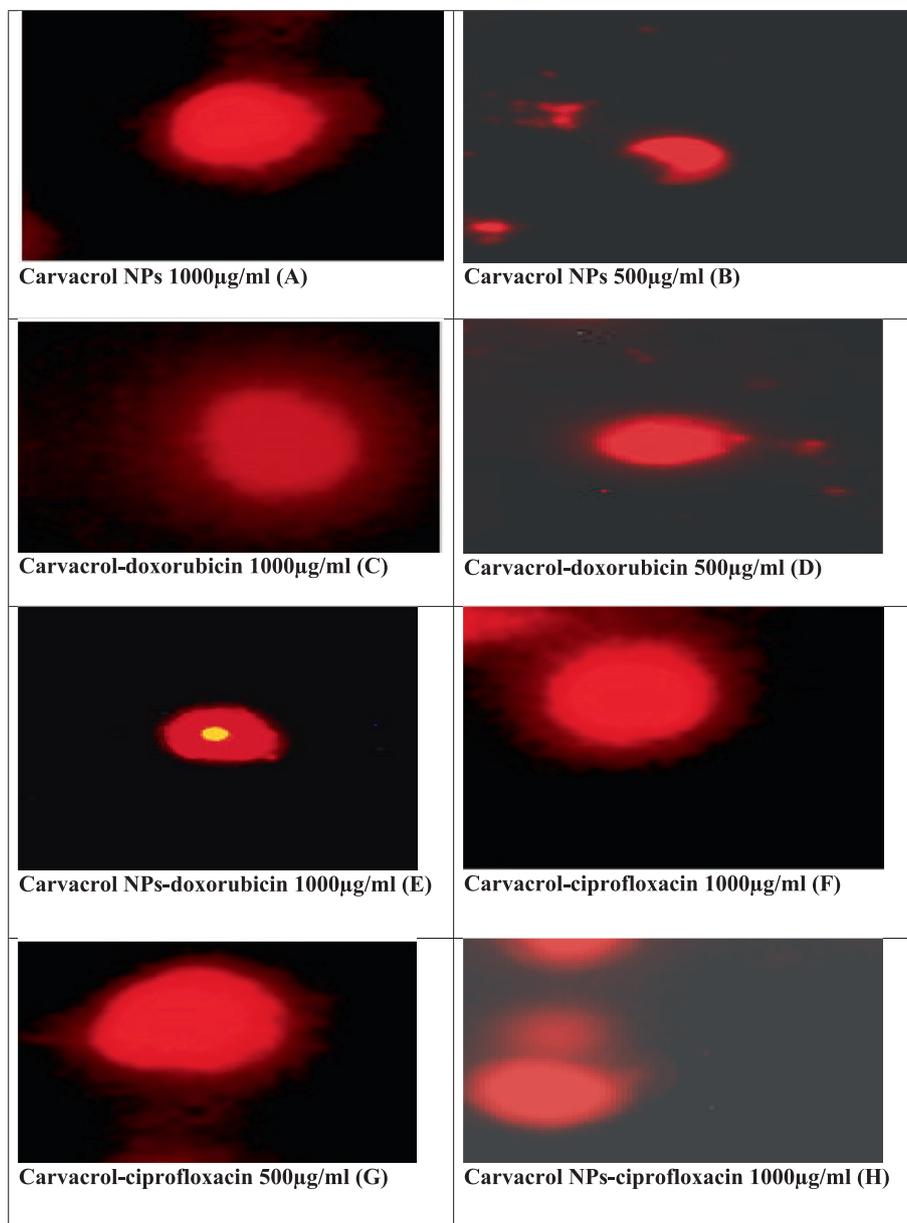
Fig. 3. (A-H) Class 1 DNA damage of carvacrol, carvacrol NPs and their combinations with Topoisomerase inhibitors on varying concentrations.

4. Discussion

Emergence and spread of antibacterial resistance thwarts the curative power of conventional antibiotics and causing major health crisis globally (Xie et al., 2023). Ciprofloxacin is commonly used Topoisomerase inhibitor to which S. aureus, E. coli and S. typhi have become resistant because of its inadequate transport across the pathogen's cell membrane (Chiang et al., 2018). The mounting threat of bacterial resistance led to the development of nanotechnology for battling intracellular microorganisms because of their extremely small regulated size, raising serum antibiotic levels and defending against bacterial resistance due to broad-spectrum antibacterial activity (Wang et al., 2017). Apart from this newly emerging CRISPR-Cas system has potential to prevent the bacterial drug resistance by identifying and targeting the genetic elements carrying genes and their transcripts (Ahmed et al., 2018).

Carvacrol is monoterpenoid phenol that has profound antibacterial activity and it alters the membrane permeability so, combination therapy using Topoisomerase inhibitors and plant-based nanoparticles may be an alternate treatment for resistant bacteria (Marinelli et al., 2018). Combination therapy is a strategy that combines two or more therapeutic drugs to achieve synergism, dose reduction and protection from bacterial resistance (Farhat and Khan 2022).

In vitro susceptibility testing was performed through disk diffusion method and inhibition zones were not observed by tested bacteria that ensured the ciprofloxacin resistance. These results are correlated with the findings of (Gupta et al., 2020). The antibacterial activity was evaluated by using agar well diffusion method and it was revealed that alone and drug combinations showed antibacterial effect and the inhibition zones were dependent on their concentrations. Meanwhile, it was observed that drug combinations increased the zone of inhibitions as compared to alone drugs. These results coincide with the study of (Abdeltwab et al., 2019).

The percentage inhibition and MIC$_{50}$ were determined through microdilution method. The percentage inhibition was increased by

increasing drug concentration and more pronounced inhibition was obtained with the drug combinations as compared to alone drugs. Carvacrol MIC_{50} was observed at concentrations 266.36 µg/ml, 355.5 µg/ml and 284.5 µg/ml while chitosan MIC_{50} was observed at 72.04 µg/ml, 70.66 µg/ml and 75.79 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively but their combination such as carvacrol-chitosan reduced the MIC_{50} to 38.28 µg/ml, 44.01 µg/ml and 44.34 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively so, the dose reduction of carvacrol was 85.71%, 87.62% and 84.41% respectively while the dose reduction of chitosan was 46.86%, 37.71% and 41.49% on *S. aureus, E. coli* and *S. typhi* respectively. These results are collaborated with the findings of (Niza et al., 2020).

Ciprofloxacin MIC_{50} was observed at concentrations 227.6 µg/ml, 175.15 µg/ml and 197.48 µg/ml but its combination with carvacrol such as carvacrol-ciprofloxacin reduced the MIC_{50} to 99.28 µg/ml, 97.47 µg/ml and 105 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively. In case of carvacrol-ciprofloxacin combination the dose reduction of carvacrol was 62.72%, 72.58% and 63.09% while the dose reduction of ciprofloxacin was 56.37%, 44.35% and 46.83% against *S. aureus, E. coli* and *S. typhi* respectively. These results are consistent with the findings of (Sobhani et al., 2017).

Doxorubicin MIC_{50} was observed at concentrations 72.48 µg/ml, 103.08 µg/ml and 103.08 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively but the combination of carvacrol-doxorubicin reduced the MIC_{50} to 52.8 µg/ml, 56.25 µg/ml and 51.88 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively. In case of carvacrol-doxorubicin combination the dose reduction of carvacrol was 80.17%, 84.17%, and 81.76% while the dose reduction of doxorubicin was 27.15%, 45.43% and 49.67% against *S. aureus, E. coli* and *S. typhi* respectively. These results correlated with the study of (Yeo et al., 2018).

Carvacrol NPs MIC_{50} was observed at concentrations 124.61 µg/ml, 303.15 µg/ml and 117.5 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively but when they were combined with Topoisomerase inhibitors such as ciprofloxacin and doxorubicin then drastically their dose was reduced. The combination of carvacrol NPs-ciprofloxacin reduced MIC_{50} to 35.8 µg/ml, 48.74 µg/ml and 35.57 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively. In case of carvacrol NPs-ciprofloxacin combination the dose reduction of carvacrol NPs was 71.27%, 83.92% and 69.72% while the dose reduction of ciprofloxacin was 84.27%, 72.17% and 81.98% against *S. aureus, E. coli* and *S. typhi* respectively. The combination of carvacrol NPs-doxorubicin reduced MIC_{50} to 20.79 µg/ml, 34.35 µg/ml and 25.32 µg/ml against *S. aureus, E. coli* and *S. typhi* respectively. In case of carvacrol NPs-doxorubicin combination the dose reduction of carvacrol NPs was 83.31%, 88.66% and 78.45% while the dose reduction of doxorubicin was 71.31%, 66.67% and 75.43% against *S. aureus, E. coli* and *S. typhi* respectively. These results coincide with previous study as described by (Rao et al., 2020).

Carvacrol nanoparticles show antibacterial effect due to presence of free hydroxyl group and delocalized electron system that play important roles by altering the ion exchange and membrane permeability. Altered permeability of bacterial membrane increases the uptake of Topoisomerase inhibitors and consequently bactericidal action along with reduced dose and resistance is obtained (Shakeri et al., 2019).

The interaction between carvacrol and chitosan combination was studied. FICI values 0.68, 0.75 and 0.74 were obtained against *S. aureus, E. coli* and *S. typhi* respectively. As FICI values were > 0.5–1.0, so they represented additive effect. These results correlated with the findings of (Pei et al., 2009). Carvacrol and ciprofloxacin combination FICI values 0.81, 0.83 and 0.90 were observed against *S. aureus, E. coli* and *S. typhi* respectively. As FICI values were > 0.5–1.0, which represented the additive effect. Carvacrol and doxorubicin combination FICI values 0.93, 0.70 and 0.69 were observed against *S. aureus, E. coli* and *S. typhi* respectively. As FICI values were > 0.5–1.0, so they represented additive effect. These results corroborate with the findings of (Batista et al., 2019).

Carvacrol NPs and ciprofloxacin combination FICI values 0.44, 0.44 and 0.48 were obtained against *S. aureus, E. coli* and *S. typhi* respectively. As FICI values were ≤ 0.5, which represented the synergistic effect. The combination of carvacrol NPs and doxorubicin FICI values were 0.45, 0.45 and 0.46 against *S. aureus, E. coli* and *S. typhi* respectively. As FICI values were ≤ 0.5 so they represented synergism. These results are consistent with findings of (Requena et al., 2019).

Genotoxicity was evaluated through Single Cell Gel Electrophoresis assay. Carvacrol nanoparticles, carvacrol-doxorubicin and carvacrol-ciprofloxacin combinations did not exhibit genetic damage from 250 to 31.25 µg/ml. At high concentrations 1000 µg/ml, 500 µg/ml genetic damage was observed where the tail length was higher than the positive control. However, carvacrol NPs-doxorubicin and carvacrol NPs-ciprofloxacin combinations did not exhibit genetic damage from 500 to 31.25 µg/ml. At high concentration 1000 µg/ml genetic damage was observed where the tail length was higher than the positive control. As carvacrol, carvacrol nanoparticles along with Topoisomerase inhibitors show genetic damage index at high concentrations that ensured their safety profile.

In previously described study dose-dependent genotoxicity of carvacrol, thymol and their combination on gastric adenocarcinoma cells was observed. Cells exposed to different concentrations of carvacrol, thymol and their combination exhibited changes in tail of DNA at high concentrations (Günes-Bayir et al., 2018).

5. Conclusion

The findings of the present study indicated that carvacrol nanoparticles not only has antibacterial effect but also showed the synergetic effect when combined with Topoisomerase inhibitors *i.e.,* ciprofloxacin and doxorubicin against *S. aureus, E. coli* and *S. typhi*. MIC_{50} of Topoisomerase inhibitor drugs was significantly decreased, when used in combination with carvacrol nanoparticles rather than used alone against the tested bacteria due to alteration in membrane permeability. Furthermore, the results proved that carvacrol NPs in combination with Topoisomerase inhibitor drugs found safe for cell DNA damage and combination therapy can be used against bacteria irrespective of their strains to resolve the drug resistance limitations and combating bacterial infections.

6. Author's contribution

Amina Akhlaq and Muhammad Ashraf designed, authored and summarized the results of experiments on the preparation and characterization of carvacrol loaded chitosan nanoparticles, the evaluation of drugs and their combinations for antibacterial activity and the comet assay. Imran Altaf determined MIC_{50} of drugs. Muhammad Ovais Omer critically analyzed the results and approved the manuscript for submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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