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CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF NEWLY SYNTHESIZED HETEROCYCLIC COMPOUND

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ABSTRACT
Present work comprises of synthesis of 3-Benzyl-2-6 bis-(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2-6-bis(2-chlorophenyl)piperidin-4-one. The structure of the synthesized compound was elucidated by spectral studies such as IR, ^{1}H, ^{13}C-NMR, Elemental analysis and Biological studies. The antibacterial activity of these derivatives was assessed with series of gram-positive and gram-negative bacteria. The antibacterial activity and the minimum inhibitory concentration (MIC) of the 3-Benzyl-2-6 bis-(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2-6-bis(2-chlorophenyl)piperidin-4-one were carried out using agar disc diffusion with various concentrations of 25µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml which results in inhibiting DNA synthesis by affecting the activity of DNA topoisomerase. The minimum antibacterial activity showed (T1) 6±0.36 mm and (T2) 5±1.21 mm diameter against *E.coil* in 75 µg/ml. MIC result of the both compounds 3-Benzyl-2-6-bis-(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2-6-bis(2-chlorophenyl)piperidin-4-one revealed that, the activity was observed between 128-512 µg/ml concentration against *Staphylococcus aureus* and *Bacillus subtilis.*

KEYWORDS: Piperidine, FT-IR, ^{1}H, ^{13}C-NMR and antibacterial activity.

1. INTRODUCTION
Piperidine is a class of heterocyclic amine consists of six-membered ring with five methylene bridges and one nitrogen unit in adjacent position $(CH_2)_5NH$.[1] The Piperidine derivatives are ubiquitous building blocks in the synthesis of pharmaceuticals and industrial applications for the production of dipiperidinyl dithiuram tetrasulfide used as accelerator. It is also used for chemical degradation reactions such as DNA sequencing in cleavage of particular modified nucleotides.[2] Piperidine derivatives are responsible for many biological activities such as analgesic, anti-hypersensitive, anti-viral, bactericidal etc.[3] Antibiotic resistance and re-emerging diseases are known to cause major medical challenges in most healthcare systems (Hogberg et al., 2010). Resistance and multidrug-resistant pathogens are spreading with extraordinary speed, globally (Hogberg et al., 2010) leading to increased mortality rates amongst patients infected (Freire-Moran et al., 2011). Piperidine scaffold has found beneficial roles in numerous pharmaceutical drugs that are currently available in the market (Perumal et al., 2014, Das and Brahmachari, 2013, Sumati Anthal et al., 2013). Alogliptin, Ritalin and Risperidone are pharmaceutically available drugs containing the piperidine nucleus that are utilized for the treatment of diabetes, improved concentration in children and reduce schizophrenia.[4] It has been reported by 2015 established new candidates with improved antimicrobial activity, against *Staphylococcus aureus* and *Aspergillus niger*. The compound antimicrobial activity and is found to fit well with the binding sites of the target protein.[5]

In present research the newly piperidin derivatives were synthesized and spectral characterized their antibacterial activity.

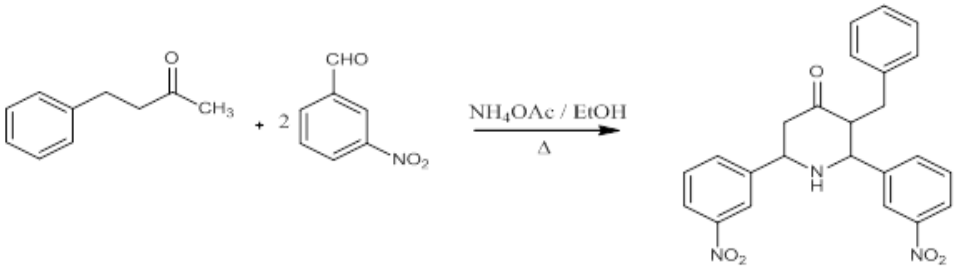
2. MATERIALS AND METHODS
All the reagents and solvents used were of laboratory grade. The melting points of the compounds were determined by open capillaries on a Thomas Hoover apparatus and are uncorrected. The purity and homogeneity of compounds were checked using TLC technique. IR spectra were recorded using KBr pellets on

Perkin Elmer 337 spectrophotometer, ^{1}H NMR were recorded on Bruker WH 500 spectrophotometer using $CHCl_3$ and DMSO as solvent.

3. EXPERIMENTAL METHODS

3.1. Synthesis of piperidine derivatives

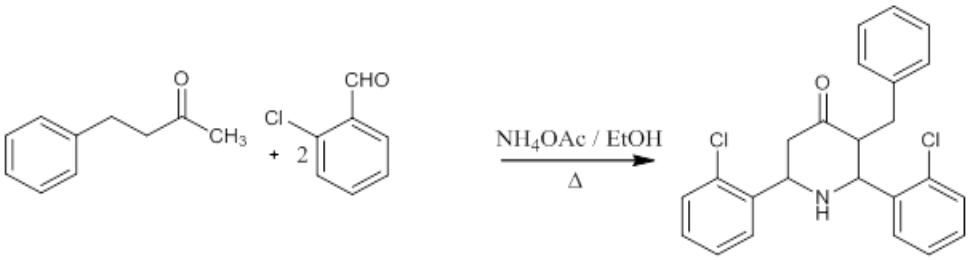
4-phenyl-2-butanone (1.4ml; 0.1 mol), ammonium acetate (4g; 0.1 mol) and 3-nitrobenzaldehyde (3.1 g; 0.02 mol) were taken in a RB flask containing ethanol (20ml). The mixture was refluxed in a water bath with occasional shaking until the colour changed into red orange. The solution was cooled and then ether (50 ml) was added. The filtered solution was transferred into conical flask and Con.HCl (5 ml) was added. A white precipitate was formed. The precipitate was washed with 5:1 ethanol:ether mixture and dried. Acetone (10 ml), liquid ammonia (5 ml) and excess of cold water were added. The precipitate was formed, filtered and dried. Then the product was recrystalised with ethanol. The product was dried, melting point is 168-170^{0}C.



3-Benzyl-2-6-bis-(3-nitrophenyl)piperidin-4-one.

3.2. Synthesis of piperidine derivatives

4-phenyl-2-butanone (1.4ml; 0.1 mol), ammonium acetate (4g; 0.1 mol) and 2-chlorobenzaldehyde (2.1 ml; 0.02 mol) were taken in a RB flask containing ethanol (20ml). The mixture was refluxed in a water bath with occasional shaking until the colour changed into red orange. The solution was cooled and then ether (50 ml) was added. The filtered solution was transferred into conical flask and Con.HCl (5 ml) was added. A white precipitate was formed. The precipitate was washed with 5:1 ethanol:ether mixture and dried. Acetone (10 ml), liquid ammonia (5 ml) and excess of cold water were added. The precipitate was formed, filtered and dried. Then the product was recrystalised with ethanol. The product was dried, melting point 170-172^{0}C.



3-Benzyl-2-6-bis(2-chlorophenyl)piperidin-4-one.

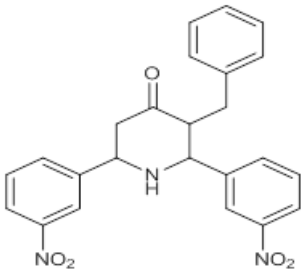
RESULTS AND DISCUSSION

Spectral Characterization

3-Benzyl-2-6-bis-(3-nitrophenyl)piperidin-4-one Yield: 87-94%; mp:168-170^{0}C. FT-IR (KBr):3326 (υN-H), 3037 (υaromatic -CH), 2921 (υaliphatic -CH), 1747 (υC=O), 1472,1439 (υC-C) cm^{-1}, ^{1}HNMR (500MHz, DMSO-d_6, δ in ppm); 7.98-7.08 (m,16 H, aromatic-H); 4.26-4.15 (d, 2H, benzylic-H (C_3 and C_5 protons); 3.34-3.36 (d, 2H, benzylic-H (C_2 and C_6 protons); 2.07 (s, 1H, NH). ^{13}C-NMR (500MHz, DMSO-d_6, δ in ppm): 207.4(>C=O), 153.9, 149.48, 133.27 -124.05, 62.45, 54.91

Based on the above spectral data the compound is identified as.

3-BENZYL-2-6-BIS-(3-NITROPHENYL)PIPERIDIN-4-ONE and the given structure as

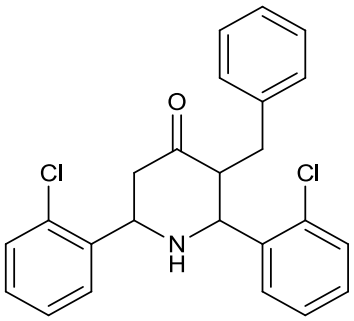


3-Benzyl-2-6-bis(2-chlorophenyl)piperidin-4-one Yield: 87-94%; mp:170-172^{0}C. FT-IR (KBr):3341 (υN-H), 3089 (υaromatic -CH), 2929 (υaliphatic -CH), 1733 (υC=O), 1472,1425 (υC-C)cm^{-1}, ^{1}H-NMR (500MHz, DMSO-d_6,δ in ppm); 7.99-7.40 (m,17H, aromatic-H); 4.14-4.24 (d, 2H, benzylic-H (C_3 and C_5 protons); 3.54-

3.55 (d, 2H, benzylic-H (C_2 and C_6 protons); 2.21 (s, 1H, NH). ^{13}C-NMR (300MHz, DMSO-d_6, δ in ppm): 206.6 (>C=O), 143.93, 136.24, 130.43 -125, 62.29, 52.22.

Based on the above spectral data the compound is identified as.

3-BENZYL-2-6-BIS(2-CHLOROPHENYL)PIPERIDIN-4-ONE and the given structure as



4. Antibacterial activity

The antibacterial activity and the minimum inhibitory concentration (MIC) of the 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6-bis(2-chlorophenyl)piperidin-4-one were carried out using agar disc diffusion (Cos et al., 2006; Abate et al., 1998) against bacterial strains which were obtained from the stock at the Department of Biotechnology. The bacterial strains used in this study were *Bacillus subtilis Escherichia coli, Staphylococcus aureus, Klebseilla sp and Pseudomonas aeruginosa.* Piperidine derivatives were dissolved in dimethyl sulfoxide (DMSO) and tested at the following concentrations, 25µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ ml.

4.1. Well Diffusion Technique

Screening of antibacterial activity was performed by well diffusion technique. The Mueller Hinton agar plates were seeded with 0.1 ml of the standardized inoculum of each test organism. The inoculum was spread evenly over plate with sterile glass spreader. The seeded plates were allowed to dry in the incubator at 37°C for 20 minutes. A standard cork borer of 8 mm diameter was used to cut uniform wells on the surface of the MHA extract was introduced in the well. Respective solvent was used as control. The inoculated plates were incubated at 37°C for 24 hours and zone of inhibition was measured to the nearest millimeter (mm).

4.2. Determination of minimum inhibitory concentration (MIC)

Different concentration (8,16,32,64,128,256 and 512µg/ml) of 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6-bis(2-chlorophenyl)piperidin-4-one were dissolved in DMSO and mixed with 50µl of nutrient both and 50µl of overnight bacterial inoculums. Nutrient broth alone was served as negative control. Whole setup in triplicate was incubated at 37°C for 24hrs in thermostat shaker. After incubation the tubes were examined by turbidity observations.

4.3. Observation of the action of 3-Benzyl-2,6-bis(3-nitrophenyl)-piperidin-4-one and 3-Benzyl-2,6-bis(2-chlorophenyl)piperidin-4-one on the membrane structure of bacteria

Different volume of NB medium, 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6- bis(2-chlorophenyl)piperidin- 4-one solution and bacterial cultures were added to 10 ml cultures to achieve final MIC concentration of compound and bacterial cultures of *B. subtilis* and *S. aureus*. Control experiment was conducted without 3-Benzyl-2,6-bis(3-nitrophenyl) piperidin-4-one and 3-Benzyl-2,6-bis(2-chlorophenyl)piperidin- 4-one. The cultures were incubated at 37°C with shaking at 150 rpm for 4 hrs and 8 hrs. The *B. subtilis* and *S. aureus* suspensions were centrifuged in sterile plastic centrifuge tubes at 8000 g for 15 min at 4°C and then were washed with saline for three times. Then the supernatant was discarded and the pellet was fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) overnight at 4°C. After the cells were dehydrated, embedded and stained, they were observed.

5. SDS-PAGE assay

B. subtilis and *S. aureus* grew on NB medium containing MIC concentration of 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6- bis(2-chlorophenyl)piperidin- 4-one. Control experiment was conducted in absence of 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6- bis(2-chlorophenyl)piperidin- 4-one. After the cultures were incubated at 37°C with shaking at 150 rpm for 2 hrs, 4 hrs, 8 hrs and 12 hrs the samples were centrifuged for 10 min at 6,000 g. The supernatant was discarded. Then 150 µl double distilled H_2O and 50 µl DTT were added to the pellet. Samples were boiled for 10 min and then 10 µL of each sample was loaded on the gel. Electrophoresis was performed at 80 V through the stacking gel (5%) and at 120 V through the separation gel (12%).

6. RESULT AND DISCUSSION

Antibiotics are the most powerful drugs in the world today since can save millions of lives. Antimicrobials are very important therapeutic agents and have been found to be clinically effective in many protozoan bacterial and fungal infections (Patel *et al.* 2010).[6] Over the past decade, fungal infection became an important complication and a major cause of morbidity and mortality in immunocompromised individuals such as those suffering from tuberculosis, cancer or AIDS and in organ transplant cases (Turan-Zitouni et al., 2005).[7] The novel antimicrobial agents continues to use as the existing antimicrobials has been limited by their relatively high risk of toxicity, pharmacokinetic problems and development of bacterial and fungal resistance resulting from the widespread use and misuse of classical antimicrobial agents.[8] Therefore antimicrobial resistant drug can be modulated by

designing new derivatives of the existing drugs. Results of antibacterial activity from 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6- bis(2-chlorophenyl)piperidin- 4-one were shown in table 1 and table 2.

Table 1: Antibacterial activity from 3-Benzyl 2,6 bis-(3-nitrophenyl) piperidin 4-one and 3-Benzyl 2,6 bis-(2-chlorophenyl) piperidin 4-one.

Name of the Species	Concentration (µg/ml)								
	Control	25 µg/ml		50 µg/ml		75 µg/ml		100 µg/ml	
		T1	T2	T1	T2	T1	T2	T1	T2
Bacillus subtilis	-	-	-	--	-	6±0.36	7±1.36	8±0.49	9±1.49
Escherichia coli	-	7±1.2	-	8±1.6	-	8±1.2	5±1.21	9±0.96	6±1.58
Staphylococcus aureus	-	7±0.36	-	8±1.6	-	9±0.89	8±1.65	9±0.69	8±1.29
Klebseilla sp	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa.	-	-	-	-	-	-	-	-	-

T1- 3-*Benzyl 2,6-bis (3-nitrophenyl)-piperidin 4-one*, **T2-** 3-*Benzyl 2,6-bis-(2-chlorophenyl)- piperidin 4-one*

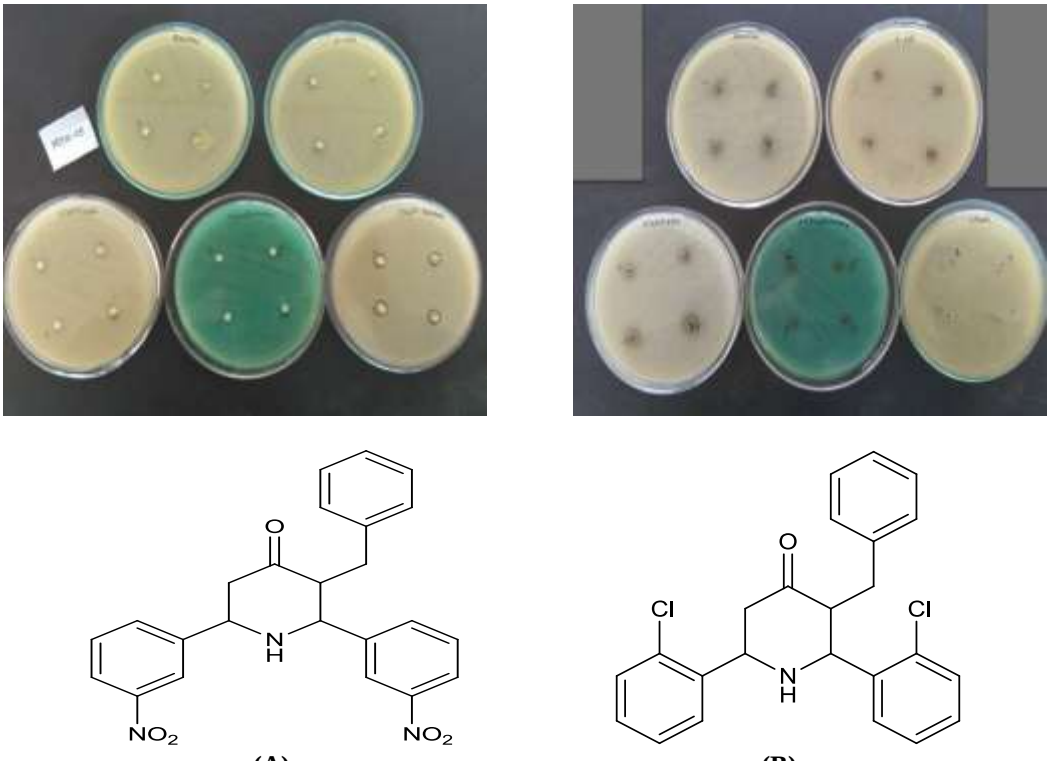
The antibacterial potential of 3-Benzyl-2,6 bis- (3-nitrophenyl) piperidin -4-one (T1)and 3-Benzyl 2,6-bis(2-chlorophenyl)piperidin-4-one (T2) reveals that, extract showed maximum antibacterial activity (9±0.69, 9±0.96 and 8±0.49 mm dia in 100 µg/ml) and (8±1.29, 9±1.46 and 6±1.58 mm diameter in 100 µg/ml) against *Staphylococcus aureus*, *Bacillus subtilis and E.Coli.* The minimum antibacterial activity showed (T1) 6±0.36 mm and (T2) 5±1.21 mm diameter against *E.coil* in 75 µg/ml (Table 1).

Table 2 showed MIC result of the both compounds 3-Benzyl 2,6-bis(3-nitrophenyl) Piperidine 4-one and 3-Benzyl -2,6-bis(2-chlorophenyl)- piperidin 4-one reveals that, the activity was observed between 128-512 µg/ml concentration against *Staphylococcus aureu* and *Bacillus subtilis.*

Table 2: Minimum inhibitory concentration values of 3-Benzyl-2,6-bis-(3-nitrophenyl) Piperidine-4-one and 3-Benzyl-2,6- bis(2-chlorophenyl)-Piperidine-4-one.

Name of the pathogens	MIC (µg/ml)
Staphylococcus aureus	256
Bacillus subtilis	256

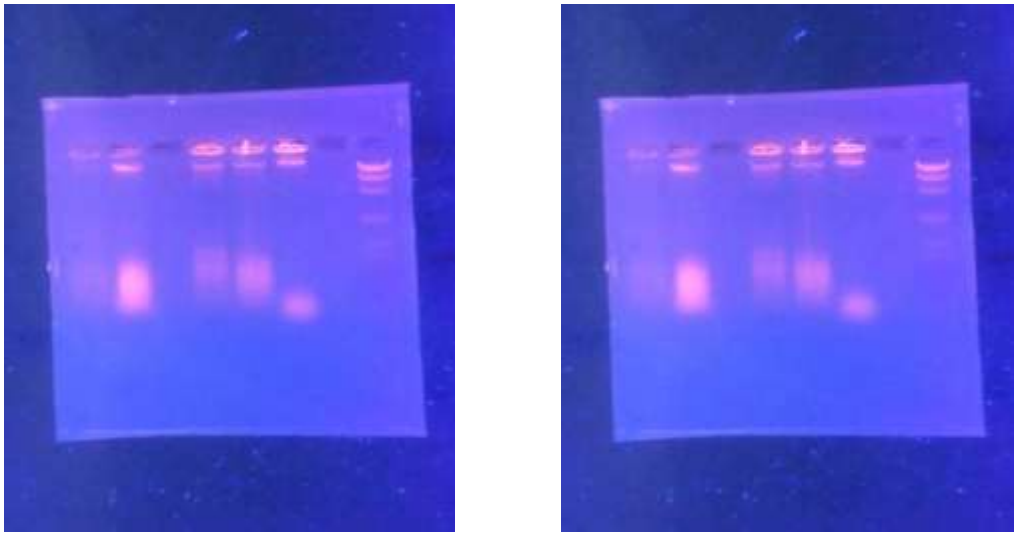
The might be due to the inhabitation of cell wall synthesis, accumulation of lysozymes or inhibition of cell multiplication. The antibacterial activity of synthesized of 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-Benzyl-2,6- bis(2-chlorophenyl)piperidin-4-one compound is represented in Fig 2.



(A) **(B)**

Fig. 2: Antibacterial activity from (A) 3-Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and (B)3-Benzyl-2,6- bis(2-chlorophenyl)piperidin- 4-one.

SDS-PAGE profiles of proteins from treated and untreated *S. aureus* and *B. subtilis* cells are shown in Figure 3. Lane 6 was the marker and control. Lane 1 and 3 were protein patterns of *S. aureus*, and *B. subtilis* and lane 4 cells treated with 3-*Benzyl 2,6- bis-(3-nitrophenyl) piperidin 4-one* and 3-*Benzyl 2,6-bis-(2-chlorophenyl)-piperidin 4-one* for 2 hrs, 4 hrs, 8 hrs and 12 hrs respectively. The protein profiles of bacteria treated with 3-*Benzyl 2,6-bis-(3-nitrophenyl) piperidin 4-one* and 3-*Benzyl 2,6- bis-(2-chloroyphenyl)-piperidin 4-one* differed from those of the control. Protein bands observed for untreated *S. aureus* and *B. subtilis* were more than the treated cells. There were less kinds and amount of bands between 66.4 KDa and 29 KDa (T1) 60.4 KDa and 28 KDa (T2) than control. The change of protein bands from (T1 approximately 66.4 kDa) in lane 2-5 and T2 showed 58.4 kDa) in lane 4 was apparent. The more time the bacteria were treated, the lower the intensities of the protein bands were observed.



(A) **(B)**

Figure 3: SDS-PAGE whole protein profiles from bacteria treated and untreated with (A) 3-Benzyl-2,6-bis-(3-nitrophenyl)-piperidin-4-one **(B)** 3-Benzyl-2,6-bis-(2-chlorophenyl)-piperidin-4-one**.**

The parasitic bacteria such as *S.aureus*, *B. subtilis* and *E.coli* have significant infections in the humans resulted in massive destruction of host tissue and life-threatening diseases. However, in this study, when exposure to 3-*Benzyl-2,6-bis(3-nitrophenyl)piperidin -4-one* and 3-*Benzyl-2,6-bis (2-chlorophenyl)piperidin-4-one* indicated that *Benzyl-2,6-bis(3-nitrophenyl) piperidin-4-one* could cause bacterial death by completely destroying proteins or partially degrading proteins and it might inhibit DNA synthesis by affecting the activity of DNA topoisomerase. The 3-*Benzyl 2,6-bis(2-chlorophenyl)piperidin-4-one* suggesting that could inhibit the growth and reproduction of *S. aureus*, and *B. subtilis*. These results suggested that membrane of bacteria would be served as an important action site for drugs. But it is still a mystery where the damage takes place. Additionally, the study showed that 3-*Benzyl 2,6 bis(3-nitrophenyl)piperidin -4-one* and 3-*Benzyl 2,6 bis(2-chlorophenyl)piperidin-4-one* had the effect on some proteins of *S. aureus* and *B. subtilis* measured by SDS-PAGE which is a powerful tool to dissociate proteins into individual chains and separate them according to their molecular weight.

SDS-PAGE is therefore an ideal technique to use for demonstrating antimicrobial effectively and has previously been used to study resistance mechanisms in bacteria (Brozel et al 1993). The SDS-PAGE results showed some protein bands of treated bacteria became low and even disappeared, suggesting that 3-*Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one* and 3-*Benzyl-2,6-bis(2-chlorophenyl)piperidin-4-one* could cause bacterial death by completely destroying proteins or partially degrading proteins. Our experiment results suggested that 3-*Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one* and 3-*Benzyl-2,6-bis(2-chlorophenyl)piperidin-4-one* might inhibit DNA synthesis by affecting the activity of DNA topoisomerase.

7. SUMMARY AND CONCLUSION

Piperidine is mainly used for chemical degradation reactions, such as DNA sequencing and also used as a base for the deprotection of Fmoc-amino acids used in solid-phase peptide synthesis.[12] To evaluate the antibacterial activity of 3-*Benzyl-2,6-bis(3-nitrophenyl)piperidin -4-one* and 3-*Benzyl-2,6-bis (2-chlorophenyl)piperidin-4-one* against S. *aureus* and *B. subtilis* and elucidate its mechanism, we studied the inhibitory effect of *S. aureus* and *B. subtilis* on bacterial growth, membranous structure and synthesis of protein and DNA. In conclusion 3-*Benzyl-2,6-bis(3-nitrophenyl)piperidin -4-one* and 3-*Benzyl-2,6-bis (2-chlorophenyl)piperidin-4-one* had antibacterial activities against *S. aureus* and *B. subtilis* by damaging the membrane and inhibiting synthesis of protein and DNA. Nevertheless, the further mechanism of interaction of 3-

Benzyl-2,6-bis(3-nitrophenyl)piperidin-4-one and 3-*Benzyl-2,6-bis(2-chlorophenyl)piperidin-4-one* with *S. aureus a*nd *B. subtilis* still need to be explored in future research studies.

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