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EVALUATION OF ANTIBACTERIAL ACTIVITY OF PHOSPHATE ESTERS BY WELL DIFFUSION METHOD

Mithilesh Kumari Gupta[1], S. K. Jadhav[2], S. A. Bhoite[1*]

[1]School of Studies in Chemistry, Pt. Ravishankar Shukla University Raipur C.G. 492010 India.

[2]School of Studies in Biotechnology, Pt. Ravishankar Shukla University Raipur C.G. 492010 India.

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***Correspondence for Author**
S. A. Bhoite
School of Studies in Chemistry, Pt. Ravishankar Shukla University Raipur C.G. 492010 India
sa.bhoite@gmail.com

ABSTRACT

Resistance in some pathogenic bacterial strains to conventional antibiotics has initiated to search for effective treatments against microorganisms. In this context antibacterial activity of mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate were studied by determining the zone of inhibition of bacterial growth against four pathogenic bacterial strains *Staphylococcus aureus* MTCC 3160, *Klebsiella oxytoca* ATCC 13182, *Bacillus subtilis* BAB 2437 and *Bacillus licheniformis* MS 17. Different concentrations of test compounds were applied against bacterial strains to examine their antibacterial activity by using well diffusion method. Gentamycin and water were taken as a positive and negative control to test the bioactivity of phosphate esters. Zone of inhibition was observed and measured in mm. Minimum inhibitory concentration (MIC) of phosphate esters against pathogenic bacterial strains was also determined.

KEY WORDS: Mono-6-chloro-2,4-dinitroaniline phosphate, di-2-methyl-5-nitroaniline phosphate, antibacterial activity, well diffusion method, zone of inhibition, MIC.

INTRODUCTION

Bacteria are extremely diverse from a metabolic standpoint and are found almost everywhere on the earth in vast numbers. They are both beneficial and pathogenic bacteria. Beneficial bacteria are involved in such diverse processes as digestion in animals, nitrogen fixation in roots of certain legumes, the decomposition of animal and plant remains. On other hand

pathogenic bacteria cause severe and often fatal disease in humans, animals and plants.[1,2] Bacteria are haploid, reproduce colony and their link with disease is appreciated. The treatment of bacterial infections is increasingly complicated by the ability of bacteria to develop resistance to antibacterial agents.[3-5] The rapid rise in the bacterial resistance to the traditional antibiotic has prompted a continuing search for new classes of compounds with novel modes of antibacterial activity.

Phosphate esters have been shown great importance due to their potential bioactivity and significant biological interest [6,7]. They have attracted the attention of medicinal chemists as chemotherapeutic agents, such as antibacterial, antifungal and antioxidants are well documented [8-10]. These are the derivatives of orthophosphoric acid which form a large family of chemical agents with biological properties that have important and sometimes unique applications for the benefit of mankind [11]. They have found a wide range of applications in areas of industrial agricultural and medicinal chemistry owing to their biological and physical properties as well as their utility as synthetic intermediates [12]. Some phosphate esters are normally considered as important pharmacological compounds [13]. Because of available antimicrobials failure to treat infectious diseases, many researchers,[14-18] have focused their works on synthesis of new biologically active compounds against microorganisms. In same context present investigation belongs to synthesis of new phosphate esters with C-N-P linkage and study of their antibacterial activity against pathogenic bacterial strains. The esters with C-N-P linkage are known as phosphoramides [19]. They have a long history in scientific literature, but it is only with their use in antiviral strategies in the early 1990s, they have attracted large consideration [20].

MATERIALS AND METHODS

Synthesis of mono-6-chloro-2,4 dinitroaniline phosphate has been done by the reaction of equimolar (1:1) quantities of 6-chloro-2,4 dinitroaniline (sigma-Aldrich) and P_2O_5 (Phosphorus pentaoxide) in benzene using the method described by cavalier [21]. Solid mass obtained was separated and water soluble part was precipitate out using $Ba(OH)_2$ solution. Thus the barium salt of monoester obtained as white solid was washed several times with distilled water having few drops of acetic acid to remove inorganic phosphate. The synthesis of di-2-methyl-5-nitroaniline phosphate was carried out according to the method described by Rudert [21] which involves the reaction of 2-methyl-5-nitroaniline (sigma-Aldrich) and $POCl_3$ (phosphorus oxychloride) in 2:1 mol ratio in benzene to give crude diester as a solid. Solid

crude diester obtained was dissolved in ammonia and recrystallized by requisite volume of hydrochloric acid, to get pure sample of di-2-methyl-5-nitroaniline phosphate. All the chemicals used were of AR grade.

Confirmation of compound:

Compounds were characterized by elemental and infrared spectral analysis at Central Drug Research Institute (CDRI), Lucknow, (U. P.) India. An IR spectrum was recorded on FTIR Perkin-Elmer Model Spectrum RX1 using KBr discs. Elemental analysis: Mono-6-chloro-2,4-dinitroaniline phosphate ($C_6H_3N_3O_7ClPBa$): Calculated C: 16,64%, H: 0.70%, N: 9.71%, Found: C: 16.32%, H: 0.69%, N: 9.68%. Infrared spectral analysis: IR (KBr): cm^{-1} 3155.33 (N-H stretching), 3380-02 (O=H Stretching), 2923.12 (C-H aliphatic aromatic stretching), 1576.70 (C=C aromatic stretching), 1268.77 (P=O stretching), 740.17, 989.47, 843.54 (P-N stretching) and 933 (C-N streching), 1941.22-2008.40 (NO_2 Streching).

Elemental analysis: Di-2-methyl-5-nitroaniline phosphate ($C_{14}H_{15}N_4O_6P$) Calculated C: 45.91%, H: 4.13%, N: 15.30%, Found: C: 45.93%, H: 4.15%, N: 15.27%. Infrared spectral analysis: IR (KBr): cm^{-1} 3326.21 (N-H stretching), 3407.42 (O=H Stretching), 2968.22 (C-H aliphatic aromatic stretching), 1513.62 (C=C aromatic stretching), 1255.43 (P=O stretching), 758.88 (P-N stretching) and 933 (C-N streching) 1832-1740 (NO_2 streching).

Collection and maintenance of Pure Culture

To study the antibacterial activity of phosphate esters, four pathogenic bacterial strains were procured from the School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur (C.G.) India. Bacterial samples were *Staphylococcus aureus* MTCC 3160, *Klebsiella oxytoca* ATCC 13182, *Bacillus subtilis* BAB 2437 and *Bacillus licheniformis* MS 17. The Pure cultures of bacteria were grown at pH 7 on nutrient agar medium (NAM) having 5 gm/ lit. NaCl, 3 gm/l beef extract 5 gm/ lit. peptones and 15 gm/ lit. agar at 37^{o}C. Stock cultures of bacteria were maintained on nutrient broth medium.

Determination of antibacterial activity by well diffusion method

To study the antibacterial activity of synthesized phosphate esters bacterial cultures maintained on nutrient agar slants were taken and aseptically inoculated in an autoclaved sterile broth plugged with sterile cotton. Broth containing respective bacteria were shacked thoroughly and incubated at 37^{o}C for 24 hours. These were designated as the working stocks and used for antibacterial studies. The antibacterial activity of synthesized phosphate esters

was assessed in vitro against four pathogenic bacterial strains using well diffusion method [22]. Petri dishes and necessary glasswares were autoclaved at 121°C and 15 lb pressure for 20 min. Nutrient agar plates were prepared by pour plate method. For agar well diffusion 100µl of the bacterial suspension was inoculated on semi solidified nutrient agar medium and spread properly. Small wells about 6 mm diameter of size were made in to semisolid nutrient agar media. Phosphate esters were screened over the range of 500, 1000, 1500, 2000 µg/ml concentrations. For this purpose requisite amount of mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate was dissolved in water and DMSO respectively. 100 µl of solution of the different concentrations was inoculated in to each well. No antibacterial activity was noted for the solvents employed in the test. Gentamycin was used as a positive control while water is used as negative control to test the bioactivity of compounds. All the petri plates were incubated at 37°C for 24 hours. Antibacterial activity of compounds was determined by measuring the diameter of zone of inhibition of bacterial growth in mm around each well. The difference in zone of inhibition at different concentrations indicates the sensitivity of phosphate esters. Each test was done in triplicate, and the mean of diameter of zone of inhibition of bacterial growth was calculated with standard error. Antibacterial activity in terms of minimum inhibitory concentration (MIC) of phosphate esters was also studied.

RESULTS AND DISCUSSIONS

Antibacterial activity

Phosphate esters showed significant antibacterial activity against all selected bacterial strains. These bacteria were screened at different concentrations ranging from 500-2000 µg/ml. There was much variation in magnitude of zone of inhibition along with primary concentrations. Results are summarized in Table 1 and 2, which shows that the diameter of zone of inhibition of bacterial growth increases with increase in concentration of synthesized phosphate esters. Characteristic differences in zone of inhibitions at different concentrations for all selected bacterial strains are illustrated in Fig 1 and 2. Diameter of zone of inhibition indicates phosphate esters exhibited moderate antibacterial activity against all selected bacteria strains. Thus phosphate esters have been found to be sensitive against all selected pathogenic bacterial strains. The antibacterial activity of phosphate esters showed little variation and excellent reproducibility of zone of inhibition for selected bacteria within 500-2000 µg/ml concentration range. Evalution of zone of inhibition was done at all concentrations and clearly shown in Fig 3 and 4. Zones of inhibition for all selected bacteria were consistently

varied with concentration. Similar observations have also been reported on the Synthesis and antimicrobial avtivity of alkyl -2-{[3-(3'-chloro-4-nitrophenyl**)**-2-oxo-3,4-dihydro-2*H*-1,3,2λ^5-benzoxazaphosphinin-2-yl] alkanoates, and some oxazaphosphinine oxides against gram positive and gram negative bacteria using paper disc diffusion method by C. S. Reddy et al.,[23, 24] 2008, 2010. Murlidhar S. Shingare et al.[15] 2006 have derived α-hydroxyphosphonate and α-acetyloxy-phosphonates and reported their antibacterial activities against gram positive bacteria by disc diffusion method using standard Streptomycin. After the evaluation of antibacterial activity, phosphate esters screened for the determination of minimum inhibitory concentration.

Minimum inhibitory concentration is the parameter which is widely used to determine the antibacterial activity and sensitivity at lowest concentration of material that inhibits the growth of an organism. Differences in MIC have been observed by well diffusion method. On lowering the concentration of test compounds the lowest concentration which is required to arrest the growth of bacteria by forming a clear zone of inhibition was regarded as minimum inhibitory concentration (MIC). Mimimum inhibitory concentration of each samples are shown in Table 3. Di-2-methyl-5-nitroaniline phosphate has been found to be more sensitive as compared to mono-6-chloro-2,4-dinitroaniline phosphate.

Table-1: Antibacterial activity of mono-6-chloro-2,4-dinitroaniline phosphate against selected bacterial strain

Concentration in µg/ml	**Zone of Inhibition (mm) with Standard Error**			
	***Staphylococcus aureus* MTCC 3160**	***Klebsiella oxytoca* ATCC 13182**	***Bacillus subtilis* BAB 2437**	***Bacillus licheniformis* MS 17**
500	11.45±0.18	12.33±0.28	10.78±0.32	10.56±0.24
1000	12.78±0.28	13.67±0.28	13.78±0.22	11.89±0.26
1500	14.44±0.34	16.33±0.33	14.78±0.36	13.89±0.26
2000	17.33±0.29	18.22±0.28	17.44±0.38	16.44±0.29

Table-2: Antibacterial activity of di-2-methyl-5-nitroaniline phosphate against selected bacterial strain

Concentration in µg/ml	**Zone of Inhibition (mm) with Standard Error**			
	***Staphylococcus aureus* MTCC 3160**	***Klebsiella oxytoca* ATCC 13182**	***Bacillus subtilis* BAB 2437**	***Bacillus licheniformis* MS 17**
500	18.11±0.26	19.11±0.35	14.22±0.36	12.67±0.33
1000	20.33±0.24	22.00±0.37	17.22±0.32	14.33±0.44
1500	22.22±0.22	24.11±0.26	19.11±0.42	17.11±0.45
2000	24.89±0.26	26.11±0.26	21.00±0.37	20.00±0.33

Table-3: Minimum inhibitory concentration of phosphate esters against selected bacteria

Bacterial strains	Mono-6-chloro-2,4-dinitroaniline phosphate		Di-2-methyl-5-nirtoaniline phosphate	
	MIC in µg/ml	Zone of Inhibition (mm)	MIC in µg/ml	Zone of Inhibition (mm)
Staphylococcus aureus MTCC 3160	400	9.44±0.18	100	11.00±0.24
Klebsiella oxytoca ATCC 13182	300	9.11±0.20	50	9.22±0.36
Bacillus subtilis BAB 2437	500	10.78±0.32	200	7.67±0.33
Bacillus licheniformis MS 17	400	8.33±0.33	400	8.11±0.35

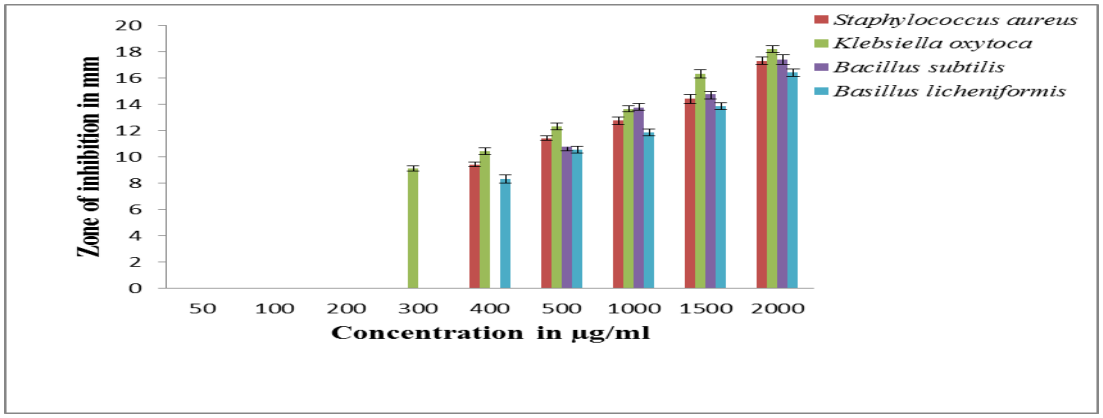

Fig-1: The graph of zone of inhibition versus concentration for MIC of mono-6-chloro-2,4-dinitroaniline phosphate against selected bacterial strains.

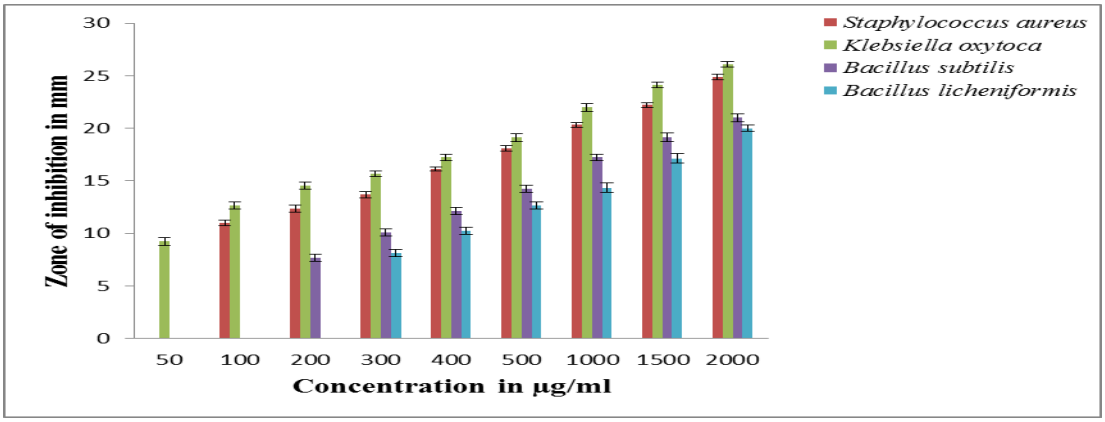

Fig-2: The graph of zone of inhibition versus concentration for MIC of di-2-methyl-5-nitroaniline phosphate against selected bacterial strains.

Photographs: Photographical representation of antibacterial activity of mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nirtoaniline phosphate against selected bacterial strains at different concentrations.

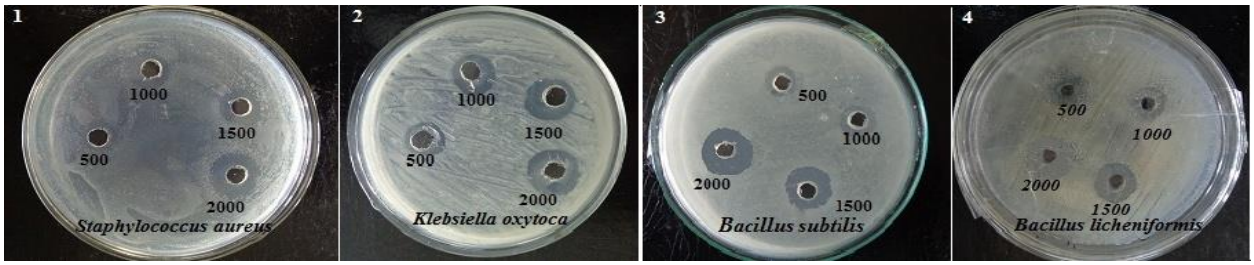

Fig-3: Zone of inhibition by mono-6-chloro-2,4-dinitroaniline phosphate over grown bacterial cultures after 24 hours at 500-2000 µg/ml concentrations by agar well diffusion method; (1) against *Staphylococcus aureus* MTCC 3160, (2) against *Klebsiella oxytoca* ATCC 13182, (3) against *Bacillus subtilis* BAB 2437 and (4) against *Bacillus licheniformis* MS 17

Fig-4: Zone of inhibition by di-2-methyl-5-nitroaniline phosphate over grown bacterial cultures after 24 hours at 500-2000 µg/ml concentrations by agar well diffusion method; (1) against *Staphylococcus aureus* MTCC 3160, (2) against *Klebsiella oxytoca* ATCC 13182, (3) against *Bacillus subtilis* BAB 2437 and (4) against *Bacillus licheniformis* MS 17

CONCLUSIONS

Mono-6-chloro-2,4-dinitroanilne and di-2-methyl-5-nitroaniline phosphate were successfully synthesized by method described earlier. They are found to be effective to inhibit the growth of selected bacterial strains. Both compounds showed a moderate antibacterial activity against selected bacterial strains. The antibacterial activity of di-2-methyl-5-nitroanilne phosphate has been found more as compared to mono-6-chloro-2,4-dinitroanilne phosphate. Both compounds exhibited better accessibility through well and easily diffused across the medium by forming a clear zone of inhibition around the well and proved to be sensitive against four selected pathogenic bacterial strains. These compounds may be useful in formulation of bactericides.

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