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Original Article

IN VITRO ANTIBACTERIAL ACTIVITY OF PHOSPHATE ESTERS SCREENED BY BROTH DILUTION ASSAY METHOD

MITHILESH KUMARI GUPTA[1], S. K. JADHAV[2], S. A. BHOITE[1]

[1]School of Studies in Chemistry, [2]School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur C. G. 492010 India
Email: sa.bhoite@gmail.com

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ABSTRACT

Objective: The present study was formulated in order to determine the novelty and the potency of the synthesized phosphate esters in terms of their antibacterial activity.

Methods: Mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate were screened for antibacterial activity against four pathogenic bacterial strains *Staphylococcus aureus* MTCC 3160, *Klebsiella oxytoca* ATCC 13182, *Bacillus subtilis* BAB 2437 and *Bacillus licheniformis* MS 17. Antibacterial activity was evaluated by the broth dilution assay method at different concentrations (50-2000µg/ml) of phosphate esters. Solutions of mono-and di phosphate esters were prepared in water and DMSO respectively. Growth of inoculums was noted in terms of optical density.

Results: Di-2-methyl-5-nitroaniline phosphate was found more active than mono-6-chloro-2,4-dinitroaniline phosphate against selected bacterial strains. The minimum inhibitory concentration (MIC) of both phosphate esters was found in the range of 25 to 50 µg/ml. Minimum bactericidal concentration (MBC) of mono-6-chloro-2,4-dinitroaniline phosphate was found in the range of 1000 to 1500µg/ml against *Staphylococcus aureus, Klebsiella oxytoca*, *Bacillus subtilis* and *Bacillus licheniformis.* Di-2-methyl-5-nitroaniline phosphate showed MBC of 500 and 400 µg/ml against *Staphylococcus aureus* and *Klebsiella oxytoca* respectively, and 1000 µg/ml against *Bacillus subtilis* and *Bacillus licheniformis***.**

Conclusion: Both the phosphate esters have exhibited significant antibacterial activity, therefore these compounds may be a good antibacterial agent.

Keywords: MIC, MBC, Antibacterial activity, Broth dilution assay.

INTRODUCTION

Antibiotics are undeniably one of the most important therapeutic discovery of the 20th century that had effectiveness against serious bacterial infections. These drugs cause a dramatic change not only in the treatment of infectious disease, but of a fate of mankind. Now day's resistance to antibacterial agents by human pathogenic bacteria is an increasingly serious worldwide health issue. The most pressing concerns are particularly with regard to the problematic human bacterial pathogens as well as fungal pathogens. They constitute a considerable portion of biomass in the earth [1-3]. Most of microorganisms can cause infectious diseases and therefore, the control of microbial growth is necessary in many practical situations. Because of available antibiotic failure to treat infectious diseases, many researchers have focused their work on synthesis of new bioactive compounds [4-6].

Staphylococcus aureus is a versatile human pathogen associated with a broad range of serious community-acquired and nosocomial diseases in humans, from minor skin and skeletal infections to severe infections such as septicemia, pneumonia [7-8]. It is a leading cause of food poisoning, resulting from the consumption of food contaminated with enterotoxins. Staphylococcal food intoxication involves the rapid onset of nausea, vomiting, abdominal pain, cramps, and diarrhoea [9-10]. Infections with this microorganism are especially difficult to treat because the strains are often resistant to one or more antibiotics, including methicillin. The increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA), and its ability to spread in the hospitals and the community, has posed a major challenge for infection control [11]. *Klebsiella oxytoca* is another important opportunistic pathogen that can cause various nosocomial and community infections, including septicemia, pneumonia, urinary tract infection, and antibiotic associated hemorrhagic colitis [12-13]. It is purported to be an etiological agent of antibiotic-associated hemorrhagic colitis (AAHC) in adults and adolescents [14-16]. Although most strains of *Bacillus subtilis* and *Bacillus licheniformis* are non-pathogenic, some variants of *Bacillus subtilis* are known to cause diseases in severely immuno compromised patients, and can conversely be used as a probiotic in healthy individuals. It rarely causes food poisoning [17-18]. *Bacillus licheniformis* are the predominant pathogenic Bacillus species and have been responsible for cases of bacteremia, septicemia, and peritonitis, food-poisoning syndrome, ophthalmitis, ventriculitis, and cerebral abscess [19-22]. It is very essential to control the growth of pathogenic strains of bacteria, by avoiding their microbial growth by means of new bacteriostatic agents.

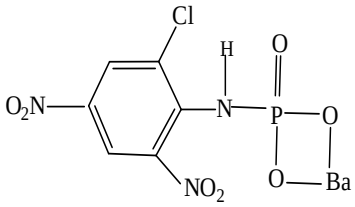
Phosphate esters are normally considered as pharmacological compounds and have attracted considerable interest on account of their diverse potential biological activity [23-24]. Some formulations of these esters are used as drugs for curing various diseases in medicine and veterinary science [25-28]. Most of non polymeric phosphate esters that occur in the cell might be considered as lead compounds for the development of drugs [29].

These esters are also used as a pro drug to enhance the water solubility of the parent drug [30]. Several studies have shown that phosphate esters have remarkable chemotherapeutic activities such as antitumor, anti-cancerous, antibacterial, antifungal, and antiviral activities [31-33]. There are very few reports on the study of bio-activity of phosphate esters. Therefore, in the present study attempt was made to synthesize phosphate esters with C-N-P linkage and evaluate their bioactivity in terms of antibacterial activity against different pathogenic bacterial strains such as *Staphylococcus aureus* MTCC 3160, *Klebsiella oxytoca* ATCC 13182, *Bacillus subtilis* BAB 2437 and *Bacillus licheniformis* MS 17.

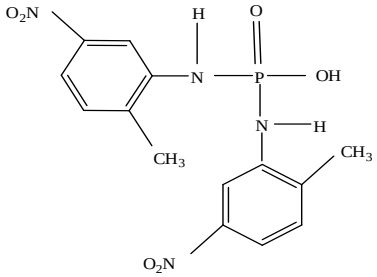
MATERIALS AND METHODS

Synthesis of mono-6-chloro-2,4-dinitroaniline phosphate was done by the method described by Cavalier which involves the reaction of 6-chloro-2,4-dinitroaniline and phosphorus pentaoxide in 1:1 mol ratio. Di-2-methyl-5-nitroaniline phosphate was synthesized by the method described by Rudert, which involves the reaction of parent compound 2-methyl-5-nitroaniline and phosphorus oxychloride in 2:1 mol ratio [34]. All the chemicals used in these experiments were

of analytical grade. The chemical structures of mono and di-phosphate ester are given below.



I. Mono-6-chloro-2,4-dinitroaniline phosphate (Ba-Salt)



II. Di-2-methyl-5-nitroaniline phosphate

To test the antibacterial activity of phosphate esters bacterial strains were procured from the collections of School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India, as *Staphylococcus aureus* MTCC 3160, *Klebsiella oxytoca* ATCC 13182, *Bacillus subtilis* BAB 2437 and *Bacillus licheniformis* MS 17.

Evaluation of antibacterial activity of phosphate esters was carried out by determining the MIC of phosphate esters for all selected bacterial strains using two different methods. The classical method involves diffusion assays in which test compound is poured on the wells of an agar plate that has been inoculated with test bacteria. During the incubation the test compound diffuses, creating a concentration gradient that produces a zone of bacterial growth inhibition [35]. In the early 1970s, automated systems were developed for assay of bacterial antibiotic susceptibility. These systems were an automated version of the classical method in which the test compound is added to the suspensions of bacterial culture to measure the bacterial growth [36].

In general the pure cultures of all selected bacterial strains were grown in Nutrient agar medium (NAM) at 37 °C. Grown bacterial cultures on nutrient agar slants were aseptically taken and inoculated into 50 ml of sterile broth. Then these were shacked thoroughly and incubated at 37 °C for 24 h. These cultures were designated as the working stock and used for antibacterial studies of phosphate esters.

Nutrient broth medium inoculated in different test tubes plugged with sterile cotton and autoclaved. Stock solutions of mono and di phosphates were prepared in water and dimethyl sulphoxide (DMSO) respectively. A set of different concentrations (25-2000 µg/ml) was designed by diluting the stock solution of test compounds in test tubes containing nutrient broth medium to test the antibacterial activity of phosphate esters employing broth dilution assay method [37]. In this method, each tube was inoculated with 100 µl of bacterial suspensions and incubated at 37 °C for 24±1 and 48±1 h. Growth of inoculums in the test tube was observed by determining the optical density (OD) at 600 nm by colorimeter. Measurements of control were carried out without the addition of test compounds. Percentage of growth inhibition of bacterial strains was calculated with respect to growth of control by the formula as given below.

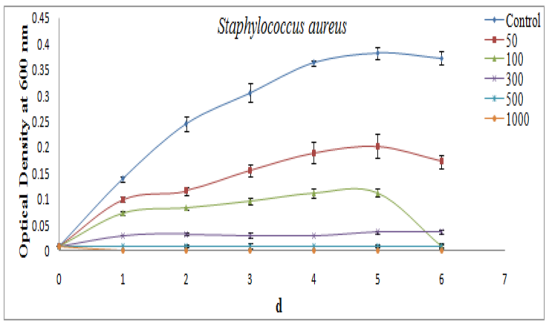
$$\text{Percentage of growth inhibition} = \frac{\text{Optical density of control} - \text{Optical density of test}}{\text{Optical density of control}} \times 100$$

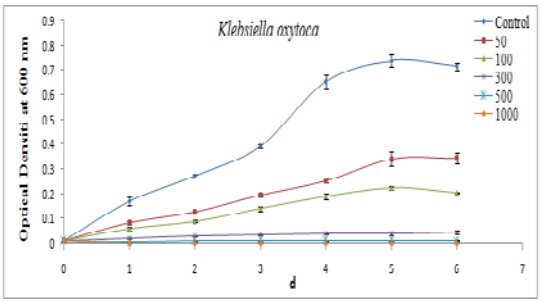
The inhibitory activity of the phosphate esters was also evaluated by well diffusion method [38, 39]. The diameter of the mean inhibition zones at different concentrations of phosphate esters was measured. Gentamycin standard antibiotic (500µg/ml) was used as a positive control and distilled water as a negative control to check the bioactivity of synthesized phosphates. After testing the antibacterial activity by this method, experiments were performed to determine the MIC of phosphate esters in nutrient broth medium by broth dilution assay method. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of material able to inhibit the growth of an organism [40].

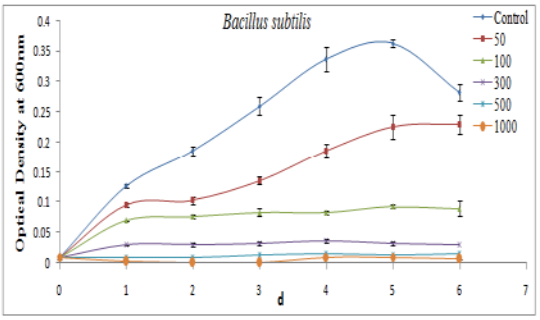
Minimum inhibitory concentration is important in diagnostic laboratories to confirm resistance of microorganisms to antimicrobial agent and also to monitor the activity of new antimicrobial agents. In this study MIC_{50}, MIC_{90} and MIC_{99} were determined which corresponds to the concentrations that inhibit 50, 90 and 99% of bacterial growth respectively. The lowest concentration with no visible growth was defined as the minimum bactericidal concentration (MBC), indicating 99.9% killing of the microorganisms [40]. All experiments were repeated three times.

RESULTS

Bacterial culture of selected bacterial strains showed typical kinetics of bacterial growth in nutrient broth media at different concentration of phosphate esters. It can be seen from the results that there was the decrease in absorbance with increase in concentration of phosphate esters. The bactericidal effect of the phosphate esters was dependent on the concentration of phosphate esters and initial bacterial concentration. The results of bacterial growth kinetics at different concentrations of phosphate esters against selected bacterial strains are shown in fig. 1 and 2.







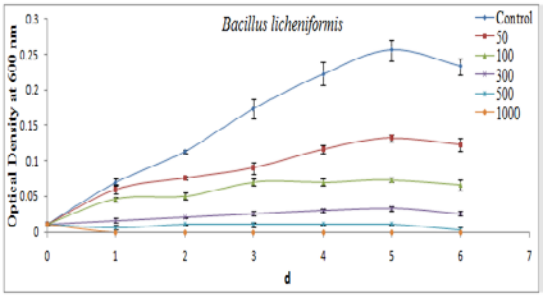
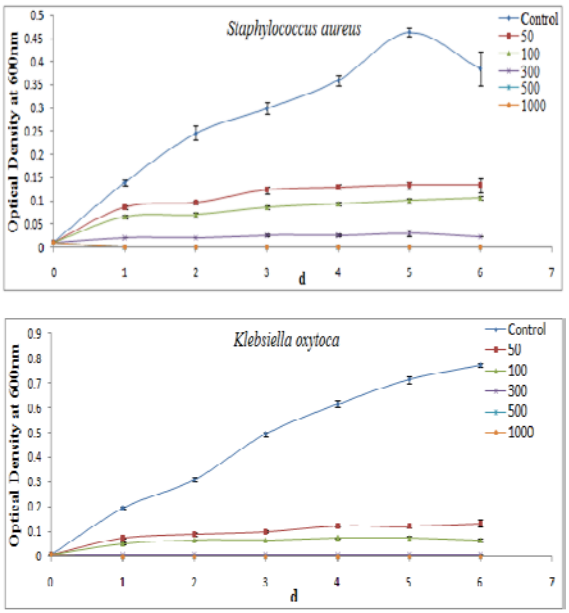


Fig. 1: Microbial growth kinetics of selected bacterial strains of Mono-6-chloro-2,4-dinitroaniline phosphate

The antibacterial activity of phosphate esters was evaluated in terms of percentage of growth inhibition. Both the phosphate esters showed significant activity against all selected bacterial strains. The minimum inhibitory concentration of both phosphate esters was found in the range of 25 to 50 µg/ml. At 25µg/ml, mono-6-chloro-2,4-dinitroaniline phosphate showed the percentage of growth inhibition as 14.33±0.59, 19.69±1.11 9.14±0.48 after 24 h and 28.30±0.69, 30.47±0.42, 16.90±1.34 after 48 h against *Staphylococcus aureus, Klebsiella oxytoca, Bacillus subtilis* respectively. But in the case of *Bacillus licheniformis* no growth inhibition was found after 24 h. The percentage growth inhibition found was 15.95±1.16 only after 48 h. Di-2-methyl-5-nitroaniline phosphate showed the higher percentage of growth inhibition at the same concentration.



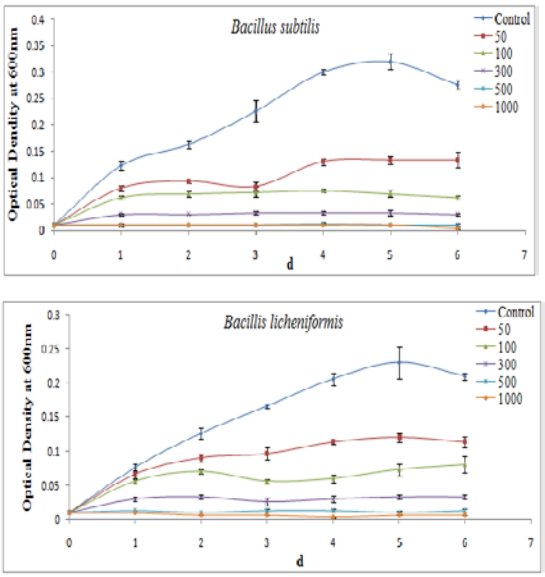


Fig. 2: Microbial growth kinetics of selected bacterial strains of Di-2-methyl-5-nitroaniline phosphate

The percentage of growth inhibition found against *Staphylococcus aureus*, *Klebsiella oxytoca, and Bacillus subtilis* was 23.73±1.55, 37.01±0.58, 18.76±1.40 after 24 h and 44.27±2.12, 48.38±0.96, 28.40±0.87 after 48 h respectively. In case of *Bacillus licheniformis* di-phosphate showed similar observation as monophosphate. Percentage of growth inhibition found was 20.45±2.27 only after 48 h. Percentage of growth inhibition of mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate are summarized in table 1,2 and table 3,4 respectively.

MIC_{50}, MIC_{90}, MIC_{99} and MBCs value for mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate against different selected bacterial strains were calculated and shown in table 5. Di-2-methyl-5-nitroaniline phosphate exhibited higher activity for all selected bacterial strains as compared to mono-6-chloro-2,4-dinitroaniline phosphate.

All selected bacterial strains depicted significant sensitivity for phosphate esters. Mono-6-chloro-2,4-dinitroaniline phosphate showed MBC at the concentration of 1000 µg/ml for *Staphylococcus aureus*, *Klebsiella oxytoca* and *Bacillus licheniformis* while at the concentration of 1500 µg/ml for *Bacillus subtilis*. Di-2-methyl-5-nitroaniline phosphate showed MBC at the concentration of 400 µg/ml for *Klebsiella oxytoca* and at the concentration of 500 µg/ml against *Staphylococcus aureus*. The MBC against, *Bacillus subtilis* and *Bacillus licheniformis* found were at the concentration of 1000 and 1500 µg/ml respectively.

Table 1: Percentage of growth inhibition of Mono-6-chloro-2,4-dinitroaniline phosphate after 24 h

Concentration in µg/ml	Percentage of growth inhibition			
	Staphylococcus aureus **MTCC 3160**	***Klebsiella oxytoca*** **ATCC 13182**	***Bacillus subtilis*** **BAB 2437**	***Bacillus licheniformis*** **MS 17**
25	14.33±0.59	19.69±1.11	9.14±0.48	----
50	28.67±1.19	50.98±0.98	18.28±0.96	13.22±1.06
100	47.61±1.21	64.62±0.21	39.34±1.56	30.16±1.59
200	66.73±1.43	76.68±1.04	60.66±1.57	51.85±4.15
300	78.50±0.88	86.32±0.98	75.91±2.14	60.32±3.18
400	85.66±0.59	94.01±0.62	85.05±2.51	73.54±2.12
500	93.61±0.85	96.67±3.81	90.86±0.48	86.77±1.06
1000	100	100	96.67±3.33	100
1500	-------	-------	100	----
2000	------	------	------	----

Table 2: Percentage of growth inhibition of Mono-6-chloro-2,4-dinitroaniline phosphate after 48 h

Concentration in µg/ml	Percentage of growth inhibition			
	Staphylococcus aureus **MTCC 3160**	***Klebsiella oxytoca*** **ATCC 13182**	***Bacillus subtilis*** **BAB 2437**	***Bacillus licheniformis*** **MS 17**
25	28.30±0.69	30.47±0.42	16.90±1.34	15.95±1.16
50	52.70±2.37	53.71±1.11	34.08±1.42	28.87±1.02
100	66.00±2.18	67.14±1.09	57.40±0.75	44.17±4.06
200	77.01±0.53	82.82±1.70	70.26±0.79	57.56±3.37
300	86.24±2.22	89.00±0.35	83.11±1.34	76.07±1.74
400	91.83±0.49	95.18±1.04	87.57±3.03	84.05±1.15
500	95.92±0.25	97.56±1.22	95.95±2.02	92.02±0.58
1000	100	100	100	100
1500	-------	-------	----	----
2000	-----	-----	-----	----

Table 3: Percentage of growth inhibition of Di-2-methyl-5-nitroaniline phosphate after 24 h

Concentration in µg/ml	Percentage of growth inhibition			
	Staphylococcus aureus **MTCC 3160**	***Klebsiella oxytoca*** **ATCC 13182**	***Bacillus subtilis*** **BAB 2437**	***Bacillus licheniformis*** **MS 17**
25	23.73±1.55	37.01±0.58	18.76±1.40	----
50	38.06±1.25	62.74±0.42	35.13±0.92	14.48±1.20
100	52.39±1.21	72.93±0.75	48.48±1.52	29.13±2.27
200	73.89±1.61	84.71±0.50	64.87±0.92	52.38±2.38
300	85.66±0.59	94.90±0.16	75.43±1.70	71.03±2.41
400	92.83±0.29	100	83.62±1.13	81.35±3.25
500	100	----	91.81±0.57	85.51±1.21
1000	----	----	100	95.24±2.38
1500	----	----	----	100
2000	----	----	----	----

Table 4: Percentage of growth inhibition of Di-2-methyl-5-nitroaniline phosphate after 48 h

Concentration in µg/ml	Percentage of growth inhibition			
	Staphylococcus aureus **MTCC 3160**	***Klebsiella oxytoca*** **ATCC 13182**	***Bacillus subtilis*** **BAB 2437**	***Bacillus licheniformis*** **MS 17**
25	44.27±2.12	48.38±0.96	28.40±0.87	20.45±2.27
50	62.32±1.20	70.91±0.91	42.75±2.18	41.16±2.64
100	72.62±2.82	78.26±1.18	56.99±1.83	58.84±2.63
200	85.79±1.14	89.19±1.28	75.69±2.74	76.52±2.73
300	92.20±0.20	96.77±0.10	81.57±0.78	85.35±2.81
400	96.20±0.10	100	87.71±0.52	85.35±2.81
500	100	----	93.86±0.26	94.19±2.91
1000	----	----	100	100
1500	----	----	----	----
2000	----	----	----	----

Table 5: MIC and MBC of phosphate esters by broth dilution assay method

Phosphate esters	Bacterial strains	MIC_{50} µg/ml	MIC_{90} µg/ml	MIC_{99} µg/ml	MBC µg/ml
Mono-6-chloro-2,4-dinitroaniline phosphate	*Staphylococcus aureus* MTCC 3160	100	500	1000	1000
	Klebsiella oxytoca ATCC 13182	50	400	1000	1000
	Bacillus subtilis BAB 2437	200	500	1500	1500
	Bacillus licheniformis MS 17	200	1000	1500	1500
Di-2-methyl-5-nitroaniline phosphate	*Staphylococcus aureus* MTCC 3160	100	400	500	500
	Klebsiella oxytoca ATCC 13182	50	300	400	400
	Bacillus subtilis BAB 2437	100	500	1000	1000
	Bacillus licheniformis MS 17	200	1000	1000	1000

The inhibitory activity of phosphate esters at different concentration was also tested by the well diffusion method. Zones of inhibition are summarized in table 6. 500µg/ml of Gentamycin was screened to check the bioactivity of phosphate esters against selected bacterial

strains. Inhibition zones found were 21.33±0.29, 29.78±0.36, 19.26±0.26, 22.45±0.24 against *Staphylococcus aureus, Klebsiella oxytoca, Bacillus subtilis* and *Bacillus licheniformis* respectively. At the same concentration of monoester inhibition zones found were 11.45±0.18, 12.33±0.28, 10.78±0.32, 10.56±0.24 against *Staphylococcus aureus, Klebsiella oxytoca, Bacillus subtilis* and *Bacillus licheniformis* respectively. Inhibition zones of di-phosphate esters found were 18.11±0.26, 19.11±0.35, 14.22±0.36, 12.67±0.33 against *Staphylococcus aureus, Klebsiella oxytoca, Bacillus subtilis* and *Bacillus licheniformis* respectively.

Table 6: Inhibitory activities of phosphate esters by well diffusion method

Phosphate esters	Bacterial strains	Concentration µg/ml	Zone of inhibition in mm
Mono-6-chloro-2,4-dinitroaniline phosphate	*Staphylococcus aureus* MTCC 3160	100	----
		400	9.44±0.18
		1000	12.78±0.28
	Klebsiella oxytoca	50	----
	ATCC 13182	300	9.11±0.20
		1000	13.67±0.24
	Bacillus subtilis	200	----
	BAB 2437	500	10.78±0.32
		1000	13.78±0.22
	Bacillus licheniformis	200	----
	MS 17	400	8.33±0.33
		1500	11.89
Di-2-methyl-5-nitroaniline phosphate	*Staphylococcus aureus* MTCC 3160	100	11.00±0.24
		500	18.11±0.26
		1000	20.33±0.24
	Klebsiella oxytoca	50	9.22±0.36
	ATCC 13182	400	17.22±0.28
		1000	22.00±0.37
	Bacillus subtilis	100	----
	BAB 2437	200	7.67±0.33
		1000	17.22±0.32
	Bacillus licheniformis	200	----
	MS 17	400	8.11±0.35
		1000	14.33±0.44

"----"shows no inhibition zone

DISCUSSION

The growth of infectious diseases and increase in bacterial resistance to traditional antibiotics has created the necessity for studies of antimicrobial activity. Therefore, many researchers have focused their studies on the synthesis of bioactive compounds and their antibacterial activity. Very limited work has been reported regarding the bioactivity of the phosphate esters till now.

In present investigation mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate were synthesized by the method described earlier [34] and screened to test their bioactivity against pathogenic bacterial strains by well diffusion and broth dilution assay methods. Standard antibiotic Gentamycin was taken as a positive control to check their bioactivity. This preliminary test was done by the well diffusion method. Standard antibiotic and phosphate esters showed better accessibility through well and easily diffused across the medium by forming a clear zone of inhibition. After testing the bioactivity by well diffusion method, MIC and MBC were determined by applying broth dilution assay method in nutrient broth medium. Different values of MIC were observed by well diffusion and broth dilution assay methods for both mono and di phosphates.

Both the phosphates were found more sensitive against gram negative bacterial strain *Klebsiella oxytoca* than gram positive bacterial strains such as *Staphylococcus aureus, Bacillus subtilis* and *Bacillus licheniformis*. This could be attributed to the structural differences of the cell wall of gram positive and gram negative bacteria. Broth dilution assay method seems to be better than well diffusion method, because in the well diffusion method, there may be chances of an increase in the relative error in the measurement of inhibition zones with a precision of 1 mm diameter. The high sensitivity of the broth dilution assay compared to well diffusion tests may be attributed to better interaction of phosphate esters with bacterial cells. MIC values were lower than the MBC values, suggesting that the phosphate esters were bacteriostatic at lower concentration but bactericidal at higher concentration. Similar study was reported earlier by Bhoite et. al.[5, 36, 41]. They have reported the synthesis of mono and di-ethylaniline phosphate and their antibacterial activity by the paper disc diffusion and broth dilution assay methods against four gram negative bacteria. The results of present compounds were found more significant than the reported earlier. Similarly Kumar *et al.* [42] have reported antibacterial activity of ethyl N-aryl-2,6-dioxo-piperid-3-ene-4-corboxylates by paper disc diffusion method against Gram-negative bacterial species *Escherichia coli, Salmonella typhimurium* and Gram-positive bacterial species *Bacillus substilis*, *Staphylococus aureus*. They have also reported minimum inhibitory concentrations (MICs) by broth dilution technique. Naga Raju et. al.[43] have reported the antibacterial activity of 6-Substituted [(1,2, 6,2)] oxathiaza-phosphonin-6-ones by Kirby-Bauer's disc diffusion method against *Staphylococcus aureus* and *Escherichia coli*.

CONCLUSION

Mono-6-chloro-2,4-dinitroaniline phosphate and di-2-methyl-5-nitroaniline phosphate have exhibited significant antibacterial activity against selected bacterial strains. Di-2-methyl-5-nitroaniline phosphate has shown more activity than mono-6-chloro-2,4-dinitroaniline phosphate. Therefore, these compounds may be useful in pharmaceutical chemistry as a good antibacterial agent.

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CONFLICT OF INTERESTS

We declare that we have no conflict of interest

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