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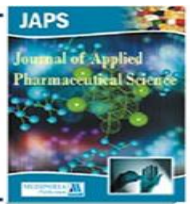

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Antibacterial activity of di-2-ethylaniline phosphate screened by paper disc diffusion method

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ABSTRACT

Antibacterial activity di-2-ethylaniline phosphate was studied against four Gram negative bacteria at 100-10000 µg/ml concentration using the paper disc diffusion method. Results indicated that the test compound, di-2-ethylaniline phosphate has demonstrated significant antibacterial activity against all selected Gram negative bacteria. Di-2-ethylaniline phosphate was found to be effective against bacteria A at lowest concentration 100 µg/ml where the zone of inhibition was 9 mm diameter in size and largest zone 24 mm diameter formed at highest concentration 10000 µg/ml against bacteria D while bacteria B and C exhibited more resistance as compared to bacteria A and D. In conclusion di-2-ethylaniline phosphate exhibited an efficient antibacterial activity.

Keywords: Di-2-ethylaniline phosphate, antibacterial activity, paper disc diffusion, zone of inhibition, MIC.

Abbreviations: mm- millimeter, DMSO-Dimethyl sulfoxide, NAM- Nutrient agar medium, MIC- minimum inhibitory concentration.

INTRODUCTION

Phosphate is important for cellular energetics, synthesis of nucleic acids, sugar phosphates, regulation of secondary metabolism and gene expression. In most plant cell cultures, phosphate is taken up readily and stored within the cells. The free intracellular phosphate is sequently converted into low molecular weight phosphorylated compounds (Zhang *et al.*, 2002). Phosphate diester linkages are found in ribose nucleic acid that play a significant role in biology (Iyer *et al.*, 2008). 3', 5'-adenosine phosphate is essential for vision, muscle contraction, neurotransmission, exocytosis and differentiation (Callahan *et al.*, 1995). The phosphoramidates synthesize phosphate esters with replacement of amide with an ester group (Mathe *et al.*, 1998; Alberg *et al.*, 1992). 1-Chloroethyl phosphates and phosphoramidates are excellent building blocks for the synthesis of novel ethylidene-linked phosphate prodrugs (Kumpulainen *et al.*, 2005). Phosphoramidases have also been found in mammalian cells and bacteria (McIntee *et al.*, 1997).

Antisense oligonucleotide therapies incorporating phosphorothioate internucleotide linkages have shown antiviral activity against HIV and Hepatitis B (Edwards *et al.*, 2011; Ushijima *et al.*, 2001). The discovery of the acute toxicity of various pentavalent organophosphorus compounds toward living species led to the development, industrial production and widespread use of phosphoric, thiophosphoric and phosphonothioic acid derivatives as biocides for animal and crop protection (Morales *et al.*, 2002; Toy *et al.*, 1987). Due to the wide importance of these esters their biological significance has been extensively investigated over the past decades (Balakrishnan *et al.*, 2004; Cox *et al.*, 1964).

The rapid emergence of multidrug resistant pathogenic bacteria has become a serious health threat worldwide (Bax *et al.*, 2000). Microorganisms are developing resistance continuously in the past several decades, so there is urgent need to discover novel antibacterial agents that could combat the antibacterial resistance (Kunin 1993). This has given an impetus for the synthesis of new group of phosphate ester and evaluation of their biological significance. Therefore the present investigation was designed to study the antibacterial activity of di-2-ethylaniline phosphate against some selected Gram negative bacteria using paper disc diffusion method.

MATERIALS AND METHODS

Collection of bacterial samples and maintenance of pure culture

Bacterial samples were procured from School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India. All collected bacterial samples were coded as A, B, C and D and pure culture of these bacteria were maintained on nutrient agar media (NAM) having 5% Peptone, 3% Beef extract, 5% NaCl, 15% Agar and pH 7.0 at 37°C. Characterization of all the selected bacteria were done by simple, Gram's staining acid fast staining methods and motility was tested by hanging drop technique (Hawker *et al.*, 1979).

Paper disc diffusion method for determination of antibacterial activity

The antibacterial activity of di-2-ethylaniline phosphate was assessed against four Gram negative bacteria. Bacterial cultures maintained on nutrient agar slants were taken and aseptically inoculated into 10 ml of sterile broth. Then broth containing respective bacteria were shaked thoroughly and incubated at 37^{o}C for 24 hours, this were designated as the working stocks which used for antibacterial studies. Di-2-ethylaniline phosphate was screened over the range of 100-10000 µg/ml concentration using paper disc diffusion method (Bauer *et al.*, 1966). For this purpose requisite amount of the di-2-ethylaniline phosphate was dissolved in DMSO to make desired concentrations 100-10000 µg/ml. 1ml of the bacterial suspension was taken and diluted in 10 ml autoclaved water and this suspension was inoculated on semi solidified nutrient agar medium by lawn culture method (Bailey *et al.*, 1974). Small autoclaved discs about 6 mm diameter size of Whatmann filter paper (No.41) were impregnated with 1ml solution of the different concentrations of 100-10000 µg/ml then these saturated paper discs were inoculated at the centre of the each petridish having bacterial lawn. In the whole investigation bacterial lawn petridishes without di-2-ethylaniline phosphate were taken as control. All the petridishes were incubated at 37^{o}C for 24 hours. The zone of inhibition around each disc was observed and measured which was indicative of the di-2-ethylaniline phosphate sensitivity at that concentration.

RESULT AND DISCUSSION

In the present investigation all the bacteria A, B, C and D were Gram negative and cocci in shape, while only one bacteria C was found positive for acid fast staining. By paper disc diffusion, zone diameter determinations of di-2-ethylaniline phosphate exhibited significant antibacterial activity against all four Gram negative bacteria. The antibacterial activity of di-2-ethylaniline phosphate showed little variation and excellent reproducibility of zone of inhibition for all selected bacteria within 100-10000 µg/ml concentration range. Evolution of zone of inhibition was observed at lowest concentration 100 µg/ml where the size of zone of inhibition was 9 mm diameter against bacteria A. Bacteria D emerged at 300 µg/ml concentration with 10 mm diameter zone of inhibition while bacteria B and C showed similar 13 mm diameter zone of inhibition at concentration 800 µg/ml, while bacteria A and D showed 15 and 16 mm diameter zone of inhibition at this concentration. Among all selected four Gram negative bacteria, different size of zone of inhibition was exhibited at 800-10000 µg/ml in increasing order (13-22 mm diameter). Similar zone formation 18 mm was obtained at 5000-8000 µg/ml, 22 mm zone was obtained at 10000 µg/ml against bacteria A. Like this bacteria B showed sequentially increase of zone of inhibition up to 8000-10000 µg/ml where the zone of inhibition was 23 mm diameter in size. Bacteria C initially started at 800 µg/ml concentration with 13 mm diameter zone and increases accordingly with concentrations, while 21 mm zone diameter formed at higher concentrations 8000-10000 µg/ml. Zone of inhibition at concentration 300 µg/ml by appearing 10 mm diameter zone and it was continuously increased up to maximum 24 mm diameter at 10000 µg/ml which was highest size of zone of inhibition in the present investigation against bacteria D (**Figure 1**). Zones of inhibition for all bacteria were consistently varied with concentration. Similar study have been reported by Ravi Sankar *et al.*, 2007 on (3a, *S*)-1-(amino acid ester)-3a, 4-dihydro-3*H*-1λ5-[1, 3, 2] oxazaphospholo [3, 4-*a*] indol-1-oxides against *Escherichia coli* (Gram negative bacteria) by the paper disc diffusion method. Mohan *et al.*, 2008 screened a series of α-aminophosphonic acid against Gram negative bacteria, *Escherichia coli* and *Klebsilla pneumoniae* by the paper disc diffusion method and found that the compounds were moderately effective against both bacteria. Similarly di-2-ethylaniline phosphate was found to be effective against Gram negative bacteria and exhibited distinct antibacterial activity.

Minimum inhibitory concentration is the widely used parameter for the determination of antibacterial activity and sensitivity at lowest concentration. In this study MIC determined at

lowest concentrations that inhibited bacterial growth and started zone of inhibition as compared to control petridishes, **Table 1**. Results indicated that the test compound found to be more sensitive against bacteria A at lowest concentration and largest zone diameter formed at highest concentration against bacteria D, while bacteria B and C showed more resistance than bacteria A and D. The present study supported the previous work where the MIC of di-2-ethylaniline phosphate was determined by broth dilution assay (Bhoite *et al*., 2012). **Figure 2** comprehensively describes the effect of different concentration against all the bacteria, insignificant differences between the zones of inhibition were observed and found comparatively similar. In spite of this the data obtained from the zone of inhibition produced by different Concentrations were found distinctly positive in zone diameter. Di-2-ethylaniline phosphate incorporated in paper disc diffusion, resulted with better resolution and reproducibility of zone of inhibition.

Table. 1: MIC of di-2-ethylaniline phosphate against four selected Gram negative bacteria.

	Control	MIC in µg/ml	Zone of inhibition
A	---	100	9 mm
B	---	800	13 mm
C	---	800	13 mm
D	---	300	10 mm

---'- indicates no zone formation, mm** - diameter of the zone in millimeter.

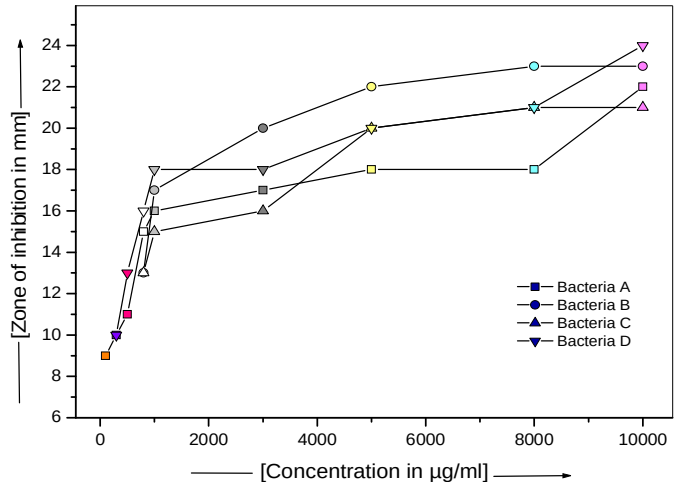

Fig. 1: Level of zone of inhibition of di-2-ethylaniline phosphate against Gram negative bacteria

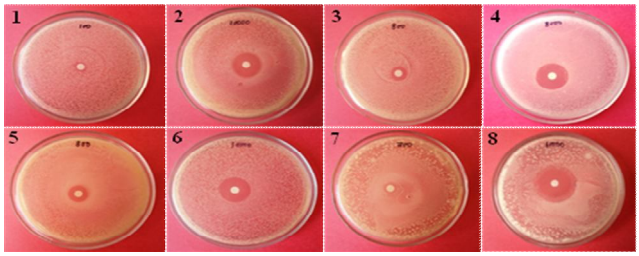

Fig. 2: Zone of inhibition by di-2-ethylaniline phosphate over grown bacterial cultures after 24 hours at initial to higher concentration by paper disc diffusion method; **1, 2-** against bacteria A at 100 and 10000 µg/ml, zone of inhibition 9 and 22 mm diameter. **3, 4-** against bacteria B at 800 and 10000 µg/ml, zone of inhibition 13 and 23 mm diameter. **5, 6-** against bacteria C at 800 and 10000 µg/ml, zone of inhibition 13 and 21 mm diameter. **7, 8-** against bacteria D at 300 and 10000 µg/ml, zone of inhibition 10 and 24 mm diameter.

CONCLUSION

Living organisms are sensitive to toxic substances and are also sensitive to such substances, which are non toxic but can cause toxicity at higher concentration. Such toxic substances effect the growth of the organism and can also alter the normal physiology. Phosphate esters are among such compounds which is effective to inhibit the bacterial growth. Di-2-ethylaniline phosphate, exhibited better accessibility through disc and easily diffused across the medium by forming a clear zone of inhibition around the disc and achieved a significant importance in microbiology showing antibacterial activity and proved to be sensitive against Gram negative bacteria. In this investigation it may be concluded that compound di-2-ethylaniline phosphate may be very useful in formulation of bactericides.

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