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Original Article

# AN ANTIMICROBIAL PHTHALATE DERIVATIVE FROM *BACILLUS CEREUS,* THE SYMBIOTIC BACTERIUM ASSOCIATED WITH A NOVEL ENTOMOPATHOGENIC NEMATODE, *RHABDITIS (OSCHEIUS) SP*

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**ABSTRACT**

**Objective:** To isolate and identify the bioactive metabolites from the culture filtrates of a bacterium (*Bacillus cereus)* symbiotically associated with a novel entomopathogenic nematode *Rhabditis* (*Oscheius*) species.

**Methods:** The bacterium was cultured in three different media and the antimicrobial activity was determined by the well diffusion assay. The ethyl acetate extract of the cell free culture filtrate was then purified by silica gel column chromatography and thin layer chromatography. Identification of the active metabolite was done with HPLC, GC-MS and LC-MS.

**Results**: The cell free culture filtrate of a nematode symbiotic bacterium showed both antibacterial and antifungal activities. Fermentation conditions were standardized and optimum antibacterial activity was observed in tryptic soy broth at 72 h of incubation at 30 °C. When the ethyl acetate extract was purified by silica gel column chromatography and thin layer chromatography, an active fraction was obtained which was subjected to HPLC analysis along with GC-MS and LC-MS leading to the identification of a major compound Bis (2-ethyl hexyl) phthalate. The compound was active against Gram positive bacteria *Bacillus subtilis* MTCC2756, *Staphylococus aureus* MTCC902, Gram negative bacteria *Escherichia coli* MTCC 2622 and fungi such as *Aspergillus flavus* MTCC277*, Candida albicans* MTCC183, *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634.

**Conclusion**: Bis (2-ethyl hexyl) phthalate was identified as one of the metabolites produced by a nematode symbiotic bacterium associated with a novel entomopathogenic nematode *Rhabditis* (*Oscheius*) species. Thus similar compounds isolated from novel entomopathogenic bacteria would pave the way for identifying new drugs for the pharmaceutical and agricultural sector.

**Keywords**: Entomopathogenic nematodes, EPN, *Rhabditis (Oscheius*) sp, Bis (2-ethyl hexyl) phthalate, Antibacterial, Antifungal.

## INTRODUCTION

Keeping the health care sector is one of the most urgent needs for humankind. One of the greatest concerns is the increasing frequency of reports of the development of resistance to drugs by many bacterial pathogens, and this is resulting in the lives of many patients being in danger. The emergence and rapid dissemination of infectious agents resistant to antibiotic treatments have emphasized the need for alternate chemicals [1]. After 50 years of intensive research on secondary metabolites from microorganisms, studies on new antibiotics and anti-tumor agents are still a hot topic of research.

*Xenorhabdus and Photorhabdus*, the symbiotic bacteria associated with entomopathogenic nematodes (EPN) steinernematidae and heterorhabditidae respectively are considered as goldmines for pharmacologically active secondary metabolites. Research has proved the antibacterial [2], antifungal [3, 4], nematicidal [5, 6] and insecticidal effects [7] of these bacteria.

Recent studies on entomopathogenic nematodes for biocontrol of insects at Central Tuber Crops Research Institute (CTCRI) paved the way for the identification of a new entomopathogenic nematode sp. namely *Rhabditis (Oscheius*) sp. [8] and their symbiotic bacteria. Sequence analysis of the D2-D3 expansion fragments of the 28S rDNA of the nematode isolate B (Accession no. CTCRI/EPN/03, NCBI deposit No HM474857) used in the present study by Deepa *et al.* [9] showed 90% similarity at the nucleotide level with that of *Rhabditis* sp. Tumian., 2007 (Accession No EU273599.1). The bacteria associated with this nematode isolate (NCBI deposit No HQ200405) showed 100% sequence similarity with *Bacillus cereus biovar anthracis, strain CI* (CP 001746) [10]. This study was designed to purify and identify the antimicrobial metabolite from this particular bacterial strain B, the crude culture of which exhibited high antimicrobial activity. This is the first report on the antimicrobial activities of Bis (2-ethyl hexyl) phthalate isolated from symbiotic bacteria associated with the entomopathogenic nematode *Rhabditis (Oscheius*) sp.

## MATERIALS AND METHODS

### Chemicals and media

All chemicals used in the study were of analytical grade. Silica gel powder, silica gel plates $F_{254}$ and the solvents such as hexane, dichloromethane, ethyl acetate, methanol, benzene, chloroform, acetone, HPLC grade methanol etc were purchased from Merck Specialities Private Limited, Mumbai, India. Microbiological media and the standard antibiotic (ciprofloxacin) discs were purchased from Himedia Laboratories Private limited, Mumbai, India.

### Test organisms and maintenance

The test organisms namely *Bacillus subtilis* MTCC2756, *Staphylococus aureus* MTCC902 and *Escherichia coli* MTCC 2622 were obtained from Microbial Type Culture Collection & Genebank, Institute of Microbial Technology (IMTECH), Chandigarh, and were subcultured on nutrient agar once in a month. The test fungi such as *Aspergillus flavus* MTCC277*, Candida albicans* MTCC183, *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634 and *Penicillium expansum* MTCC 2006 were also obtained from IMTECH and maintained on potato dextrose agar.

### Isolation of bacteria from the entomopathogenic nematodes

One nematode isolate of *Rhabditis (Oscheius*) sp. (Accession no. CTCRI/EPN/03, NCBI deposit No HM474857) is selected from 65 isolates maintained at CTCRI. The bacterial strain B was isolated from the third stage infective juveniles of the nematode using the following protocol. The nematodes were surface sterilized by putting them in streptomycin solution (5000 units/ml) for one hour

and washing it in distilled water thrice for 20 min, 10 min and 5 min respectively. These were transferred to nutrient broth and kept in shaker overnight. The nutrient broth was streaked on nutrient agar to get pure culture and it was subsequently subcultured once in two weeks and stored. For the consecutive studies, the bacterium was taken from this culture collection maintained in the CTCRI laboratory.

**Screening the bacterial culture filtrate for antimicrobial activity**

Newly subcultured bacteria were inoculated into 100 ml of Tryptic Soy Broth (TSB) and kept at 30 °C and 150 rpm overnight. This culture (with an optical density of 1.5) was used to inoculate 400 ml of TSB and incubated for 72 h in the same conditions. The optical density of the culture was checked. The culture was centrifuged at 10,000 rpm for 10 min at 4 °C to get cell free culture filtrate. It was concentrated to 5 ml with a rotary evaporator and stored at-20 °C for antimicrobial assay.-20 °C

**Assay of antibacterial activity**

The test bacteria maintained on nutrient agar were inoculated into 10 ml of nutrient broth and incubated at 37 °C for 18 h. This culture was used to inoculate on Mueller Hinton Agar (MHA) plates with a sterile cotton swab. Wells was punched (6 mm diameter) in the agar and filled with 50 µl of samples. Commercial antibiotic Ciprofloxacin (1 µg/ml, Himedia) was used as positive reference standard to determine the sensitivity of the strains. Plates were incubated at 37 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone. The mean of 3 values was recorded.

**Assay of antifungal activity**

Using a cotton swab, the potato dextrose agar surfaces were uniformly swabbed with the fungal cultures and wells were punched (6 mm diameter) in the agar and filled with 50 µl of samples. Commercial fungicide Bavistin (100 µg/ml) was used as positive reference standard to determine the sensitivity of the strains. The treatments and control were each replicated three times. After 48 h in the dark at 25 °C, the area of the inhibition zone was calculated based on the average of two diameters measured in two perpendicular directions.

**Standardization of fermentation of the bacteria for maximum activity and extraction of metabolites with an organic solvent**

The mother culture was prepared by inoculating the bacteria into 2 sets of 250 ml flasks each containing 100 ml of Tryptic Soy broth (TSB), Luria Bertani broth (LB) and Nutrient broth (NB) and kept at 30 °C and 150 rpm for 24 h incubation. This 24 h mother culture (with an optical density of 1.5) was used to inoculate 2 flasks each containing 400 ml each of TSB, LB and NB respectively. Fermentation was allowed to carry out for different time intervals such as 24 h, 48 h, 72 h, 96 h and 120 h. The culture was centrifuged at 10,000 rpm for 15 min at 4 °C and to the supernatant; half the volume of ethyl acetate was added. The flask was swirled slowly and allowed both fractions to separate out. To the aqueous fraction, again half the volume of ethyl acetate was added and allowed the fractions to separate out completely. The organic fraction was concentrated with a rotary evaporator and the concentrate was dissolved in 1 ml of methanol. This was assayed for antibacterial and antifungal activities.

**Purification of the bioactive metabolites from the organic fraction**

The initial studies of the purification of the organic fraction were carried out with thin layer chromatography (TLC). The crude organic fractions were spotted on TLC plates (Silica gel 60 $F_{254}$, Merck). The different solvent systems such as Hexane: Ethyl acetate (80:20), Hexane: Ethyl acetate (60:40), Ethyl acetate: Methanol: water (100:13.5:10) were tried. The developed TLC plate was visualized at 254 nm (short UV) and 365 nm (long UV).

**Column chromatography**

The 72 h TSB culture broth (30 l) obtained after centrifugation was extracted twice with ethyl acetate and concentrated under vacuum to yield a crude extract (10 g), which was then subjected to silica gel column (60 cm long, and 3.0 mm in diameter)` for further purification. The column was eluted with a gradient of solvent mixtures of increasing polarity starting from a non polar solvent such as hexane**,** followed by mixtures of dichloromethane in hexane (5%-100%), ethyl acetate in dichloromethane (1%-100%), finally with methanol/ethylacetate (1%-100%). 25 ml fractions were collected and were concentrated using a rotary evaporator. The purity of different fractions was checked by TLC (Silica gel 60 $F_{254}$, Merck) and similar fractions were pooled and assayed for antibacterial and antifungal activities. The fractions showing strong antimicrobial activity were further subjected to preparative TLC in benzene: hexane (3:1) solvent system to increase the purity.

**Identification of the active substance by analytical methods**

**Liquid Chromatography/Mass Spectophotometry (LC/MS)**

In order to identify the active compound, LC/MS was carried out on C18 column (100x4.6 mm, 3 um) with 100% methanol as the mobile phase. The flow rate was adjusted as 1 ml/min and the mass range was 50-450 amu with positive mode of ionization.

**Gas Chromatography/Mass Spectophotometry (GC/MS)**

GC/MS analysis was carried out in a Varian CP 3800 interfaced to a Saturn 2200 mass spectrometer. The capillary GC column used was 30 mm x 0.5 mm in dimension with 100% dimethyl polysiloxane as the packing material. The conditions were as follows: electron impact mode at an ionizing voltage 70 eV, scan range 40-600 amu, helium as the carrier gas at a constant flow of 1 ml/min and injection volume of 1 µl. The oven temperature was programmed from 100 °C to 270 °C (Isothermal for 1.5 minutes with an increase of 4 °C/min to 150 °C, then 5 °C/min to 270 °C, ending with 20 min isothermal at 270 °C).

**Confirmation of the compound by HPLC**

The suspected standard compound was purchased commercially (TCI chemicals, Chennai) and run an HPLC (C18column, 4.6X250 mm) along with the sample for confirmation of the identity of the molecule.

**RESULTS AND DISCUSSION**

**Screening the bacterial culture filtrate for antimicrobial activity**

In the present study, an attempt was made to purify the bioactive secondary metabolites from symbiotic bacteria associated with an entomopathogenic nematode. During the initial screening for the antimicrobial activity of the 72 h concentrated cell free culture filtrate, significant activities were recorded against the fungus *Aspergillus flavus* (10 mm diameter) and the bacteria *Bacillus subtilis* and *Staphylococcus aureus* (11 mm diameter each). The bacteria started to produce bioactive compounds from the end of exponential phase and reach its maximum in the stationery phase.

**Standardization of fermentation of the bacteria for maximum activity and extraction of metabolites with an organic solvent**

When the bacterial fermentation was standardized in three different media namely TSB, LB and NB for different time intervals starting from 24 h to 120 h, the maximum antibacterial activity was recorded in TSB medium at 72 h. This was followed by LB at 48h and very less activity was recorded in the case of NB (fig. 1. a-c). The antifungal activity was also shown to be the maximum in the case of TSB media at 72 h of fermentation (fig. 2. a-c).

Recently Fang *et al.* [13] performed a comparison of TSB, LB, NB and other modified media in enhancing the antibiotic activity and found that TSB medium was found to be the optimum medium for biomass and antibiotic activity. Our results also confirmed the previous reports by Maxwell *et al.* [14] and Ji *et al.* [15] about the high productivity of TSB medium. Chen [16] suggested that the different media used may contribute to the different levels of activity expressed. There is a good correlation between the antibacterial and antifungal activity of the ethyl acetate fraction, so the same agent, which is ethyl acetate extractable, may be responsible for both activities as suggested by Chen [16].

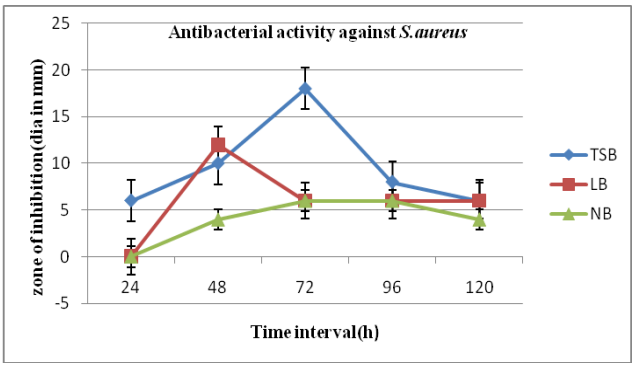


**Fig. 1(a): The antibacterial activity of the three crude organic fractions against *S. aureus***

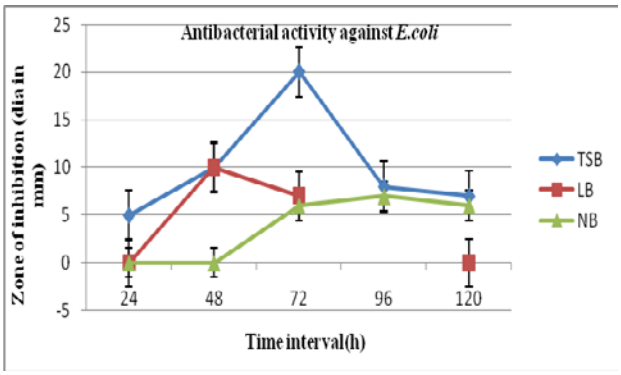


**Fig. 1(b): The antibacterial activity of the three crude fractions against *E. coli***

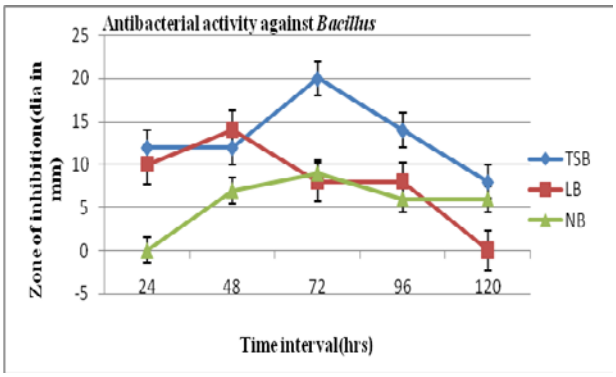


**Fig. 1(c): The antibacterial activity of the three crude fractions against *B. subtilis***

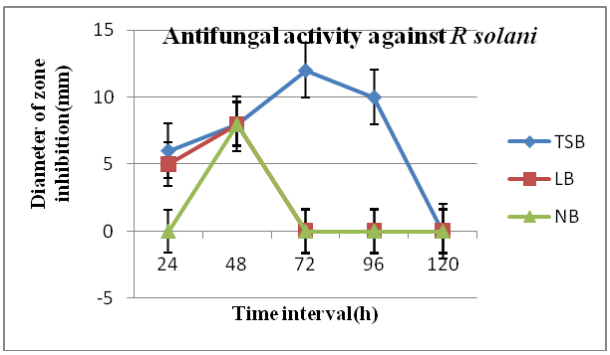


**Fig. 2(a): The antifungal activity of the three crude fractions against *R. solani***

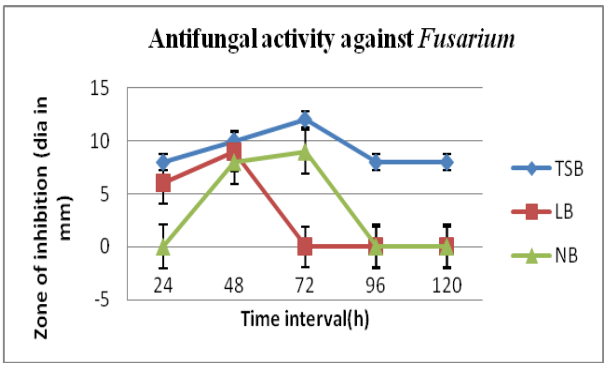


**Fig. 2(b): The antifungal activity of the three crude fractions against *Fusarium oxysporum***

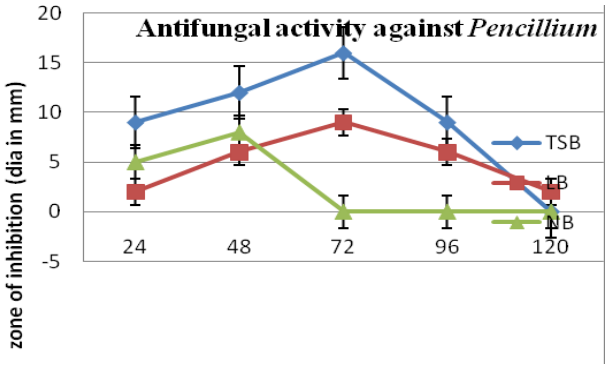


**Fig. 2(c): The antifungal activity of the three crude fractions against *Penicillium expansum***

**Purification of the bioactive metabolites from the organic fraction**

After standardization of the different media and time intervals, bacterial fermentation in tryptic soy broth (TSB) for 72 h was chosen because of its maximum antibacterial as well as antifungal activities. So this fermentation process was repeated several times to yield about 10 g of active oily organic residue from 30 l of culture broth for further purification by chromatographic methods. In order to get an idea about the number of compounds in the crude organic fraction and their polarity, an initial thin layer chromatogram was run with solvent systems such as hexane: ethyl acetate (80:20), hexane: ethyl acetate (60:40), ethyl acetate: methanol: water (10:1.35:0.1). Separation was found to be better when ethyl acetate: methanol: water (10:1.35:0.1) was used as a solvent.

**Silica gel column chromatography**

Initially the column was washed with hexane, followed by mixtures of dichloromethane (DCM) in hexane (5%-100%). The TLC was carried out with benzene: hexane (3:1) as the solvent system and similar fractions were pooled and concentrated.

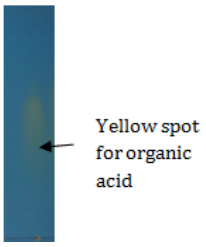


**Fig. 3: Positive result for organic acid**

This was again subjected to preparative TLC to remove the impurities. The 75% to 100% DCM fraction eluted from the column showed significant antibacterial activity against *B. subtilis and S. aureus* and antifungal activity against *A. flavus, C. albicans, F. oxysporum* and *R. solani* (table 1). This fraction gave positive result

for the test for organic acid by dipping the chromatogram in a solution of 5 mg Bromo cresol green in 25 ml ethanol and 0.25 ml of 0.1M NaOH. A yellow spot was obtained on a blue background (fig.3).

**Table 1: The antimicrobial activity of the purified fraction against different test organisms**

| Name of the test organism | Zone diameter (mm) for 50 mg/well | Standard antibiotic |
|---|---|---|
| *Bacillus subtilis* | 12 | Ciprofloxacin (1µg)-21 mm |
| *Staphylococus aureus* | 12 | Ciprofloxacin (1µg)-19 mm |
| *E. coli* | 11 | Ciprofloxacin (1µg)-20 mm |
| *Aspergillus flavus* | 10 | Bavistin (100 µg)-25 mm |
| *Candida albicans* | 11 | Amphotericin (5 µg)-23 mm |
| *Fusarium oxysporum* | 10 | Bavistin (100 µg)-16 mm |
| *Rhizoctonia solani* | 11 | Bavistin (100 µg)-19 mm |

Values represent the mean of three replications

**Identification of the active substance by analytical methods**

**Liquid Chromatography/Mass Spectophotometry (LC/MS)**

From the LC-MS data, it was clear that the major compound was having a mass of 391.3 a. m. u (fig. 4)

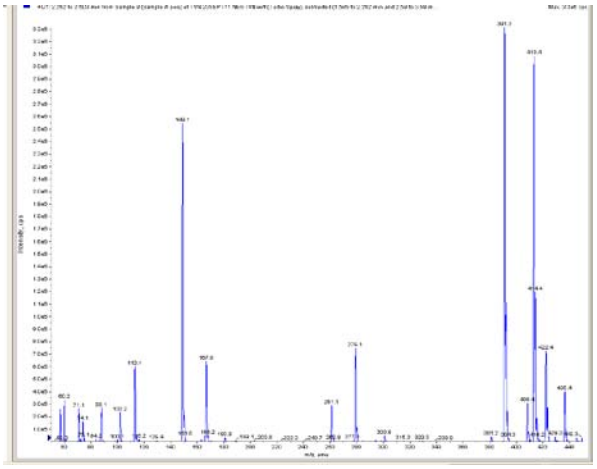

**Fig. 4: The Q1 MS of the major peak (391.3 amu)**

**Gas Chromatography/Mass Spectophotometry (GC/MS)**

Partial purification by column chromatography followed by GC-MS and LC MS studies led to the identification of Bis (2 ethyl hexyl) phthalate (fig. 5) as a major antimicrobial compound.

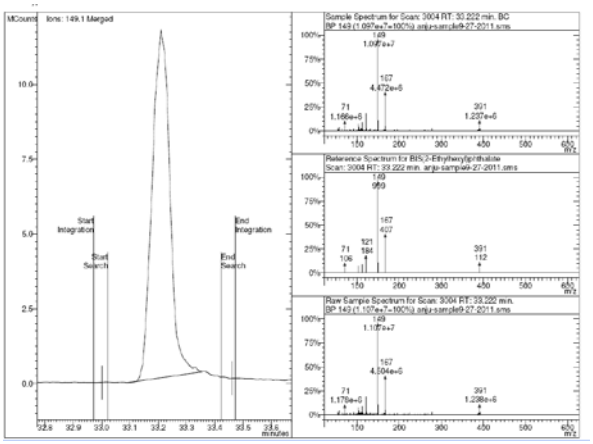

**Fig. 5: The GC spectra of Bis (2-ethyl hexyl) phthalate**

**Confirmation of the identity of the molecule by HPLC**

HPLC analysis of the purified compound showed that it eluted at a retention time of 6.3 which was comparable with that of standard (Retention time 6.23) confirming the identity of the molecule (fig. 6 and 7).

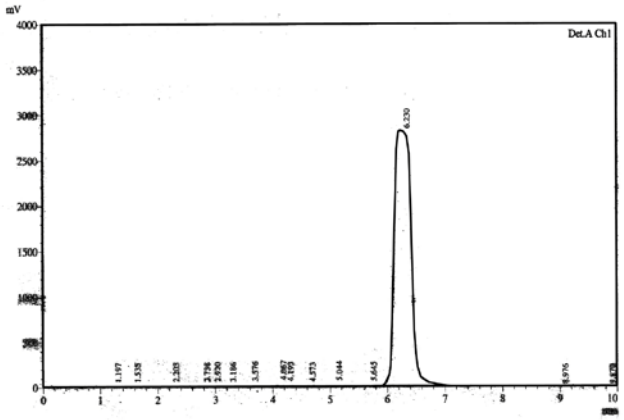


**Fig. 6: The HPLC profile of the standard**

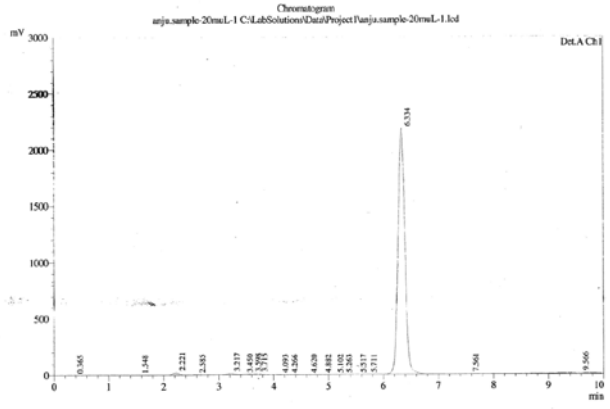


**Fig. 7: The HPLC profile of the sample**

Bis (2-ethyl hexyl) phthalate was identified as one of the metabolites produced by a nematode symbiotic bacterium associated with a novel entomopathogenic nematode *Rhabditis* (*Oscheius*) species. Phthalate compounds are petrochemicals used as plasticizers or solvents in a variety of industrial products. But many phthalate derivatives have been isolated from plants, fungi and bacteria. The first report on Bis (2-ethylhexyl) phthalate from microbial origin

was by Uyeda *et al.* [17] in *Streptomyces sp.* Bis (2-ethylhexyl) phthalate has also been reported as a bioactive compound from terrestrial and marine organisms including *Streptomyces bangladeshiensis* [18], *Streptomyces mirabilis* strain NSQu-25[19] *Pseudomonas sp.* PBO1 [20], marine algae [21-24], fungi [25] and *Nocardia levis* [26]. In the present study, this compound showed good antibacterial activity against *B. subtilis, S. aureus* and *E. coli* and antifungal activity against *A. flavus, C. albicans, F. oxysporum* similar to the reports by Kavitha *et al* [26] in *Nocardia levis.* Al Bari *et al.* [18] also reported that this compound shows a zone diameter of 15 mm and 13 mm for *B. subtilis, S. aureus* respectively at a concentration of 30 mg/disc similar to the present study. This compound is considered as pro inflammatory agent in some studies [27, 28]. The same compound was isolated from the plant *Aloe vera* and was found to have antileukemic and antimutagenic effects [29].

## CONCLUSION

Entomopathogenic nematodes (EPN) are well-known as biological control agents and are found to be associated with symbiotic bacteria which can produce a wide range of bioactive secondary metabolites. This study reports the antimicrobial activities of Bis (2-ethylhexyl) phthalate from a symbiotic bacterium associated with the entomopathogenic nematode *Rhabditis* (*Oscheius*) species. Identification of the compounds reported here is preliminary and further structural analyses need to be performed to ascertain the identity of the compounds and to evaluate its potential as a drug molecule in the pharmaceutical and agriculture industry.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTERESTS

No conflict of interest declared.

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