ABSTRACT

Bacteria belonging to the family Brevibacteriaceae are ubiquitous Gram positive organisms that are responsible for the feet odor and cheese aroma. *Brevibacterium mcbrellnerii* is a relatively new member belonging to Brevibacteriaceae. In the current manuscript we discuss isolation of biologically active metabolites from *Brevibacterium mcbrellnerii*. Two aromatic esters were isolated from *Brevibacterium mcbrellnerii* by “Bioassay guided fractionation strategy” and identified as di-(2-ethylhexyl) phthalate and dibutyl phthalate by chemical characterization using biophysical techniques. The phthalate compounds show broad spectrum antibacterial activity and mosquito larvicidal activity. Mosquito larvicidal activity has been attributed to inhibition of acetylcholinesterase enzyme activity. These compounds were found to be cytotoxic in multiple cell lines causing cell cycle arrest in G1 phase.

**Keywords:** *Brevibacterium mcbrellnerii*, di-(2-ethylhexyl) phthalate, dibutyl phthalate, anti-bacterial, Mosquito larvicidal, cytotoxicity, cell cycle arrest

INTRODUCTION

*Brevibacterium mcbrellnerii* is the member of genus *Brevibacterium* of Brevibacteriaceae family (Roux and Raoult, 2009). *B. mcbrellnerii* is aerobic, non sporulating, Gram positive rod to coccoid shaped and grows in saline environment. This distinct species was initially isolated from clinical sample (McBride et al., 1993), but can also be isolated from soil. A few biologically active metabolites have been isolated from this genus viz., 1, 6-phenazinediol 5, 10-dioxide (Iodinin) (Podojil and Gerber, 1967), 6-Hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinesdimethanol showing (Choi et al.,
2009), polyhydroxy butyrate (PHB) (Kiran et al., 2014). The aim of our present study was to isolate, characterise and determine toxic compounds from *Brevibacterium mcbrellneri*.

Two aromatic esters with anti-bacterial, larvicidal and cytotoxic properties di-(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP), were isolated whose chemical structures were determined through analysis of NMR and ESI-HRMS data. These compounds were isolated for the first time from this source. In recent years, phthalates have been isolated from strains of bacteria and fungi collected from diverse environments such as water, soils, plants and sediments. The most common fatty foods such as milk, butter, and meat were reported to have DEHP and other phthalates as contaminants, with a special mention about its toxicity at higher concentrations (Heinemeyer et al., 2013). Both DEHP and DBP have been previously reported as secondary metabolite produced from various sources like brown algae, *Undaria pinnatifida* and *Laminaria japonica*, green alga, *Ulva* sp., red algae *Bangia atropurpurea* (Chen, 2004). DEHP has been individually isolated from *Streptomyces bangladeshensis* (Al-Bari et al., 2005), *Penicillium olsonii* (Amade et al., 1994), *Alchornea cordifolia* (Mavar-Manga et al., 2008), *Aloe vera* (Lee et al., 2000), *Euphorbia cyparissias* and *Euphorbia seguieriana* (Toth-Soma et al., 1993) etc., while DBP has been reported from *Streptomyces nasri*, *Streptomyces melanosporofaciens* (Lee, 2000), *Streptomyces albidoflavus* (Roy et al., 2006). In our present study both DEHP and DBP were found to have potent antibacterial activity, mosquito larvicidal activity due to acetylcholinesterase inhibitory activity and cytotoxicity due to cell cycle arrest in G1 phase.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Nutrient Broth (Cat # M002, Himedia, India), Nutrient agar (Cat # M001, Himedi, India), Tryptone Soya Broth (Cat # LQ508, Himedia, India), Tryptone Soya Agar (Cat # M593, Himedia, India), Potato dextrose broth (Cat # M403, Himedia, India), Potato dextrose agar (PDA) (Cat # M096, Himedia, India), Osmium tetroxide (Cat # O5500, Sigma-Aldrich, USA), Fetal bovine serum (FBS) (Cat # 10270106, Thermo Fisher, USA), Dulbecco's modified Eagle's medium (Cat # D5648, Sigma-Aldrich, USA), Sodium pyruvate (Cat # S8636, Sigma-Aldrich, USA), Glutaraldehyde (Cat # G7776, Sigma-Aldrich, USA), 2, 2-dimethoxypropane (Cat # D136808, Sigma-Aldrich, USA), Ethyl acetate (Cat # 38311, SDFCL, India), n-Hexane (Cat # 20387, SDFCL, India), Silica gel (Cat # 109385, Merck, India), Potassium bromide (Cat # P0838, Sigma-Aldrich, USA), DTNB (Cat # D8130, Sigma-Aldrich, USA), Acetylthiocholine iodide (Cat # A5751, Sigma-Aldrich, USA), CDCl3 (Cat # S8636, Sigma-Aldrich, USA), 2, 2-dimethoxypropane (Cat # D190764, Sigma-Aldrich, USA), Isopropanol (Cat # 190764, Sigma-Aldrich, USA), Isopropanol (Cat # P4170, Sigma-Aldrich, USA), Doxorubicin (Cat # D1515, Sigma-Aldrich, USA), Triton-X 100 (Cat # T8787, Sigma-Aldrich, USA), RNase A (Cat # R6148, Sigma-Aldrich, USA).

**Microbial cultures and maintenance**

**Bacterial cultures**

The antibacterial evaluation was carried out against Gram positive bacteria; *Staphylococcus epidermidis* (MTCC 435), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), and Gram negative bacteria; *Pseudomonas aeruginosa* (MTCC 741), *E. coli* (MTCC 443) and *Klebsiella pneumoniae* (MTCC 618). *Brevibacterium mcbrellneri* MTCC (3913) was chosen for isolation of biologically active compounds.

**Fungal cultures**

The antifungal evaluation was carried out against *Saccharomyces cerevisiae* (MTCC 36), *Candida albicans* (MTCC 227), *Aspergillus flavus* (MTCC 277), and *Aspergillus niger* (MTCC 282) (Xie et al., 2016).

All the bacterial and fungal cultures were procured from Microbial Type Culture Collection, Institute of Microbial Technology.
(MTCC, IMTECH) Chandigarh, India. The bacterial stock cultures were maintained on nutrient broth and agar and fungal cultures on potato dextrose broth and agar. Sub culturing was done at regular intervals and stored at 4 °C (Holt, 1975).

**Mosquito larval cultures and maintenance**

The early 4th instar larvae of *Aedes aegypti* were selected for evaluation of mosquito larvicidal activity. The larvae were collected and fed with a mixture powdered yeast and dog biscuits. These were maintained in insectaries till the emergence of adults. The colony was photo periodically maintained at 14 L: 10 D (hours) and with relative humidity 80 ± 5 % at 28 °C (Kumar et al., 2010). Adult mosquitoes were fed with blood for egg maturation. The eggs were allowed to hatch in an enamel bowl lined with Whatman filter paper filled with distilled water. The pupae formed were separated and transferred to insectary for the emergence of adults (da Silva et al., 2016).

**Mammalian cell culture**

The MTT cell proliferation was assessed against the cell lines HeLa (ATCC; CCL-2), A549 (ATCC; CCL-185), MCF-7 (ATCC; HTB-22), DU145 (ATCC; HTB-81), CHo (ATCC; CCL-61), HEK293 (ATCC; CRL-1573), NIH3T3 (ATCC; CRL-1658) were procured from the American Type Culture Collection (ATCC). These cell lines were grown and maintained in Dulbecco's modified Eagle's medium with non-essential amino acids, 10 % fetal bovine serum (FBS), 1 mM sodium pyruvate, in humidified atmosphere containing 5 % CO₂ at 37 °C (Esumi et al., 2004).

**Preparation of culture for microscopy**

*Brevibacterium mcbrellneri* was Gram stained and observed under light microscopy at 100x resolution. For scanning electron microscopy the cells were collected by centrifugation, fixed with 2.5 % glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) for 2 hrs at room temperature and post fixed for 2 hrs in 1 % osmium tetroxide. Cells were then washed twice with cacodylate buffer and retained by filtration in Millipore filters (0-20 mm diameter). After giving ethanol wash, filters containing the cells were dried (Haggis and Phipps-Todd, 1977), covered with cover slip and finally coated with gold, giving a layer 40 nm thick. The cells were observed with a scanning electron microscope Model 3400N SEM (Hitachi, Japan).

**Bacterial culture for extraction of secondary metabolites**

The well grown single colonies of *Brevibacterium mbrellneri* were inoculated in subculture agar slant containing trypotone soya agar medium (gm/Lt): pancreatic digest of casein 15.0; enzymatic digest of soya bean 5.0; sodium chloride 5.0; agar 15.0; at pH 7 for 7 days at 37 °C. The obtained grown agar slant was served to inoculate 500 ml Erlenmeyer flask, each containing 100 ml of the medium. The culture medium was cultivated in the static position for 7 days. After harvesting, the resultant biomass, including the medium, was centrifuged at 10,000 rpm for 20 min at 4 °C. The biomass was separated and the remaining filtrate was extracted using ethyl acetate (1:1 v/v), and collected aqueous ethyl acetate was concentrated in-vacuo in rota-evaporator at 45 °C to afford 9.0 g as brown oily crude extract (Futamura et al., 2001). The obtained unique brown organic extract was applied for further biological screening. According to the TLC monitoring of the crude extract solvent system for further purification was identified.

**Purification of biologically active compounds**

Isolation of active compounds was carried out using solvent extraction method (Chen and Huang, 2016). For isolation of active toxic compounds, the crude extract was chromatographed in a column packed (2.5 i.d. x 50 cm) with silica gel (60-120 mesh) as stationary phase and hexane: ethyl acetate as mobile phase. The polarity was gradually increased to elute the mixture of compounds present in crude extract. The eluted fractions were TLC monitored and the biologically active fractions were further chromatographed...
to obtain two compounds. The compound isolated, from active subfractions were identified by chemical characterization on the basis of $^1$H NMR and $^{13}$C NMR, FT-IR, ESI-MS, and HRMS analysis.

**Chemical characterization**

The compound purity and structure were confirmed using the following biophysical techniques.

**Fourier Transform Infrared Spectroscopy (FT-IR)**

Thermo Nicolet Nexus 670 Spectrometer was used for FT-IR analysis. Potassium bromide was used as beam splitter, the infrared (IR) with the wavelength of 4000 cm$^{-1}$ to 400 cm$^{-1}$, was used as a radiation source at mid IR region (Majzner et al., 2013). DTGS potassium bromide with resolution of 4 cm$^{-1}$ was used as a detector.

**Nuclear Magnetic Resonance Spectroscopy (NMR)**

Tetramethylsilane (TMS) was used as the internal standard on a 300, 500 MHz spectrometer for $^1$H NMR and $^{13}$C NMR spectral recordings respectively. The conditions maintained for recording $^1$H NMR were TMS at 0.00 ppm, CDCl$_3$ at 7.26 ppm, where as for $^{13}$C NMR were CDCl$_3$ at 77.0 ppm, DMSO at 39.43 ppm (Morcombe and Zilm, 2003).

**Electrospray Ionisation Mass Spectrometry (ESI–MS)**

Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, UK) was used for recording mass spectra (Sridhar et al., 2014). Using autosampler, the samples were introduced into the mass spectrometer with acetonitrile as the mobile phase at 0.2 ml/min. The ESI capillary voltage was maintained at $+4.0$ kV, Nitrogen was used as the desolvolation gas at 150 °C. The data was acquired using Masslynx software (version 3.2).

**Electrospray Ionisation High-Resolution Mass Spectrometry (ESI-HRMS)**

Exactive Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used to record electrospray ionization high resolution mass spectra, in positive ion mode (Nagaveni and Prabhakar, 2015). Spray voltage was maintained as 4000 V and capillary voltage as 30 V at 250 °C. Using Xcalibur software (Thermo Scientific) data was acquired.

**Biological evaluation**

**Anti-bacterial activity**

The minimum inhibitory concentrations (MIC) were recorded against *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*. In brief 96-well plates were distributed with Nutrient broth and the compounds in different concentrations (150 μg/mL to 0.5μg/mL). 50 μL microbial suspensions were seeded into each well (0.5 MacFarland standards) to make the final volume of 200 μl with culture broth. The plates were incubated for 24 hrs at 37 °C. The lowest concentration, at which the visible inhibition was recorded, was considered as MIC. Streptomycin and Benzylpenicillin were used as positive controls.

**Larvicidal bioassay/Dose-response bioassay**

Based on the preliminary screening results, different concentrations ranging from 25 to 200 ppm were prepared for DEHP and 25 to 1 ppm for DBP were used. The compounds were subjected to dose response bioassay against 4th instar larvae of *Aedes aegypti* larvae. Dead larvae were counted after 24 h of exposure, and the percentage mortality was reported from the average of triplicates (Govindarajan et al., 2016).

**Acetylcholinesterase activity**

Acetylcholinesterase inhibitory activity was performed according to Ellman’s method (Tao et al., 2016) with modifications. The 4th instar larvae of *Aedes aegypti* were homogenised in phosphate buffer (0.1M; pH=7) and centrifuged at 8000 rpm for 30 min at 4 °C. Supernatant was collected and used as enzyme source. Different concentration of the compounds (30 μl) each was added into 96 well microplate along with 30 μl of supernatant and 40 μl of phosphate buffer (0.1 M;
and incubated for 1 hour. 50 μL each of 0.6 mmol/L DTNB and 1.5 mmol/L acetylthiocholine iodide were added and OD was recorded at 412 nm. Triplicates were maintained.

**MTT cell proliferation assay**

The cell lines were seeded in 96-well plates in 200 μL aliquots. The test compounds in different concentrations were added and incubated at 37 °C for 24 hrs. Doxorubicin was used as positive control (Prata-Sena et al., 2016).

**Flow cytometry analysis of nuclear DNA**

To analyze the nuclear DNA content (Patel et al., 2016), CHO cells (2 × 10^8 cells) were treated with pure compounds for 24 hrs at concentrations 150, 175, 200 μg/ml for DEHP and 10, 15, 30 μg/ml for DBP. This is followed by washing the cells twice with ice-cold PBS buffer, harvesting, fixing with cold 70 % ethanol in PBS and storing at −20 °C for 30 min. After fixation, the cells were incubated with Triton-X 100 (50 μg/mL), RNase A (0.1 mg/mL), at 37 °C for 30 min, stained with propidium iodide (50 μg/mL) for 30 min and then measured for nuclear DNA content using Amnis flowsight imaging flow cytometer from M/S Millipore with INSPIRE software.

**RESULTS AND DISCUSSION**

*Brevibacterium mcbrellneri* appears as rod shape in fresh culture and coccoid shape in old culture as described previously (McBride et al., 1993). They are non-spore forming. Both the forms are Gram-positive and were single or sometimes in pairs under light microscope. They appeared as irregular rods or in V shape when observed under the scanning electron microscope (Figure 1A and 1B). The bacilli were 5-7 μm long and 3-4 μm wide. The cocci measured around 3 μm in diameter. This is in comparison with *B. massiliense* (Roux and Raoult, 2009). The *B. mcbrellneri* shows Gram positive nature without producing spores that are irregular, short, straight rod measuring 0.4-1.4 mm in length and 0.3-0.5 mm in diameter.

Crude extract and fractions were checked for anti-bacterial activity to identify the fraction that has to be fractionated further, to isolate the biologically active compounds (Figure 1C). After the crude extract was tested for bioactivity, it was subjected to column chromatography with non-polar n-hexane and polar ethyl acetate as solvent system. The n-hexane fraction of the crude extract furnished a mixture of compounds that were eluted using a polarity gradient of solvent system. The fractionation process was monitored using TLC analysis. The fractions that exhibited similar TLC profiles were pooled and antimicrobial property of the fractions was evaluated. Fraction showing the best activity was again subjected to column chromatography to isolate bioactive molecules. A pale yellow transparent liquid (compound 1), was isolated along with a colourless transparent liquid (compound 2). The chemical structures of the 2 purified compounds were then elucidated on...
the basis of FTIR, NMR and Mass spectrometry.

**Compound 1**

The FTIR spectrometric analysis of compound 1 (Figure 2A) showed characteristic absorbance at 1728 cm$^{-1}$, representing carbonyl band and at 1072–1250 cm$^{-1}$, signifying C-O band. This data determines the presence of aromatic ester in the isolated molecule. The aromatic signals between 7.72 (m) and 7.53 (m) ppm, on the 1H-NMR spectrum (Figure 2B) have coupling constants as predicted for protons at the ortho-substituted ring. Signal at 4.24 (m, 4H) ppm is assigned to a methylene group adjacent to the ester alcohol group. With the available chemical shifts at different peaks, we could determine the possible fragment structure recorded in the spectroscopy.

The 13C NMR spectrum (Figure 2C), confirmed the symmetry of the molecule, exhibiting the expected 12 carbon resonances (δ 10.6, 13.7, 22.6, 23.4, 28.6, 30.1, 38.4, 67.6, 128.4, 130.5, 132.1, 167.3) assigned as two quaternary, three methane and five methylene carbons with two methyl groups. This data is analogous to 1H NMR data and helped in providing the Carbon backbone of the molecule. By comparison of the recorded 1H and 13C-NMR data to those published in literature (Amade et al., 1994), compound 1 was identified as di-(2-ethylhexyl) phthalate (DEHP). The mass spectrum of the expected molecule was analysed and found to be 391 (M+1)$^+$ (Figure 2D). The HRMS was recorded and calculated by using Mass centre software) database (Figure 2E), and obtained peak at m/z ratio at 391.2857 was found to match with 391.2842, which corresponds to C$_{24}$H$_{39}$O$_4$. Based on FTIR, NMR and MS data the compound was identified as di-(2-ethylhexyl) phthalate (DEHP).

**Compound 2**

The FTIR spectrum showed a characteristic absorption frequency at 1727 cm$^{-1}$ and this can be attributed to the ester carbonyl group stretching vibration (Figure 3A). The band at 1598 cm$^{-1}$ and 1588 cm$^{-1}$ corresponds to the $\text{–CH–}$ stretching for aromatic nucleus. A characteristic absorption at 1122 cm$^{-1}$ and 1072 cm$^{-1}$ are attributed to C-O stretching. This data could determine the functional group present in the molecule and could predict that aromatic ester derivative might be present in the isolated molecule. The compound was then subjected to 1H NMR (Figure 3B) analysis in 500 MHz spectrometer with CDCl$_3$ as solvent system which displayed an up field triplet at δ 0.97 for methyl protons. The multiplets at δ 1.47 (4H) and δ 1.74 (3H) represent methylene protons. A downfield triplet at δ 4.3 (4 H), represents methylene protons. The doublet of doublets at δ 7.53 (2 H) and d 7.73 (2H) indicate aromatic protons. This was followed by 13C NMR analysis (Figure 3C) in 500 MHz spectrometer with CDCl$_3$ as solvent system. It displays eight signals accounting for sixteen carbon atoms. Quartet at δ 13.3 is assigned to – 5’methyl carbon atoms. The triplets at δ 18.8, δ 30.2 and 65.0 represent methylene carbons. The downfield doublets observed at δ 128.84 and δ 130.5 represent aromatic carbon atoms. A singlet at δ 132.05 indicates tetra substituted aromatic carbon atoms. The most downfield singlet at δ 167.2 is assigned to carbonyl carbon atoms. This data provides the Carbon backbone of the isolated molecule. The mass spectrum (Figure 3D) of the expected molecule was analysed and found to be 279 (M+1)$^+$ This information provides the molecular mass of the expected molecule. The HRMS (ESI) (Figure 3E), was recorded and relative intensity vs. m/z ratio was calculated as 278.1597. This was found to match C$_{16}$H$_{23}$O$_4$ = 279.1590. This provides the molecular formula of compound. By comparison of 1H and 13C NMR data to those published in literature (Roy et al., 2006) and based on FTIR and MS data the isolated compound was identified and confirmed to be dibutyl phthalate.
Figure 2: Chemical characterization of Di-(2-ethylhexyl) phthalate: A) FT-IR spectra showed peaks at 2958, 2929, 2860, 1729, 1462, 1380, 1266, 1070, 1039, 741, 651 cm⁻¹. B) ¹H NMR spectra showed peaks at δ 7.72-7.69 (m, 2H), 7.53-7.50 (m, 2H), 4.24-4.21 (m, 4H), 1.45-1.32 (m, 16H), 0.92 (t, 12H). C) ¹³C NMR spectra showed peaks at δ 10.6, 13.7, 22.6, 23.4, 28.6, 130.5, 132.1, 167.3. D) MS(ESI): (m/z) = 391 (M⁺)+. E) HRMS (ESI) (M⁺)+ m/z calcd for C₂₄H₄₀O₄ = 391.2857, found = 391.2842.
Figure 3: Chemical characterization of dibutyl phthalate: A) FT-IR spectra showed peaks at 2960, 2874, 1722, 1265, 1199, 1069, 741, 651 cm\(^{-1}\). B) \(^1\)H NMR spectra showed peaks at δ 7.73-7.70 (m, 2H), 7.53-7.50 (m, 2H), 4.3 (t, 2H), 4.10-4.08 (d, 1H), 2.08-2.02 (m, 1H), 1.47-1.33 (m, 4H), 0.97 (t, 7H). C) \(^{13}\)C NMR spectra showed peaks at δ 13.3, 18.8, 132.0, 167.2. D) MS(ESI): (m/z) = 279 (M+1\(^+\)). E) HRMS (ESI) (M+1\(^+\)) m/z calcd for C\(_{16}\)H\(_{23}\)O\(_4\) = 278.1597, found = 279.1590.
The compounds were effective against both Gram positive and Gram negative bacteria; the MIC was compared with the standards Penicillin and Streptomycin (Figure 4A). Anti-bacterial activity of isolated ester derivatives di-(2-ethylhexyl) phthalate and dibutyl phthalate. DEHP was most effective against *S. epidermidis* with MIC of 9.37 µg/ml followed by *S. aureus* (18.75 µg/ml). It was equally effective against *B. subtilis* along with *E. coli* showing an MIC of 37.5 µg/ml. It showed activity against *P. aeruginosa* and *K. pneumoniae* at 75 µg/ml. DEHP’s effect against *S. epidermidis* was comparably close to the standard antibiotics. DBP had maximum effect on both *B. subtilis* and *S. epidermidis* showing activity at 18.75 µg/ml. It had equal activity against *E. coli*, *P. aeruginosa* and *S. aureus* (MIC= 37.5 µg/ml). It showed activity against *K. pneumoniae* at 75 µg/ml.

Previously there are reports of antimicrobial activity on DEHP isolated from Calotropis gigantean showing MIC at 32 µg/ml against *B. subtilis* and *Sarcina lutea* and with negligible antifungal property (Habib and Karim, 2009). There are also reports of antimicrobial activity on DBP isolated from Streptomyces albidxflavus showing MIC on *E. coli* 53 µg/ml, *B. subtilis* 84 µg/ml, *S. typhi* 76 µg/ml, *S. cerevisiae* 92 µg/ml, *A. niger* 98 µg/ml, Curvularia pallescens 117 µg/ml (Roy et al., 2006). DEHP and DBP were also evaluated for their toxicity against the larvae of *Aedes aegypti*. The compounds caused mortality in 4th instar of *Aedes aegypti* after 24 hrs (Figure 4B and 4C). Mosquito larvicidal activity against the di-(2-ethylhexyl) phthalate and dibutyl phthalate). The highest mortality was observed in DBP, with LC50 at 1.62 ppm. It caused 100 % mortality at 25 ppm. DEHP showed LC50 at 47.65 ppm. The effect of different concentrations, ranging from 50-300 µg/ml was observed on AChE enzyme activity, and the % inhibition was recorded (Figure 4D and 4E). Acetylcholinesterase activity against di-(2-ethylhexyl) phthalate and dibutyl phthalate). For DHEP and DBP the IC50 was recorded as 173.29±4.8 µg/ml and 138.92±6.6 µg/ml respectively. This is the first report on DEHP and DBP showing AChE inhibition, against mosquito larva *Aedes aegypti*. Previously there are reports of these compounds showing AChE inhibition, against embryonic Zebra fish (Xu et al., 2013).

The effect of DHEP and DBP on the proliferation of normal and cancer cell lines, with doxorubicin as positive control, was evaluated by MTT cell proliferation assay (Figure 5A). DBP showed cytotoxicity against CHO with IC50 = 36±8.6 µg/ml whereas DEHP could show mild cytotoxicity with IC50 = 193±5.4 µg/ml. DBP was also effective against HEK 293 with IC50 = 19.77±10 µg/ml which is more toxic than the standard doxorubicin with IC50 = 21.80±8.8 µg/ml. DHEP and DBP showed IC50 = 33.46±7.7 µg/ml and 31.23±8.7 µg/ml respectively, against DU-145. DHEP and DBP were active against MC7 with IC50 = 46.55±0.1 µg/ml and 32.43±3.6 µg/ml respectively. From the above study we could say that both DHEP and DBP showed cytotoxicity properties in normal cells and also to all the cancer cell lines in a dose-dependent manner. Cytotoxic activity of DBP has been reported against A549 cells (Hsu et al., 2011). Based on the MTT cell proliferation assay, we carried out FACS studies to verify the toxic effect of isolated compounds on cell cycle and DNA content in CHO cell line (Figure 5B). In both the compounds tested concentrations revealed significant inhibition of cell proliferation at the G1/S boundary leading to an increased accumulation of cell population in G1 phase of the cell cycle, with relative significant depletion of cell number in S phase in CHO cells. At the G1 gate, cell population was noticed to increase from 20 % to 28 % with a corresponding decrease of 21 %-22 % of cells in S-phase indicating that both the compounds at all the exposed concentrations caused arrest of cells at the G1 checkpoint and inhibited further DNA synthesis of cells. FACS analysis (Figure 5) demonstrated the presence of 46 % of cells in G1 phase; 25.3 % in S-phase, 26.4 % in G2/M and 1.9 % of cells in SubG0/G1 with DMSO after 24 hr exposure. Previously there
are reports on DEHP showing cell cycle arrest at G1 phase of growth in HaCaT cells (Peropadre et al., 2015) and that DEHP could show apoptosis without cell cycle arrest HePG2 cells (Lyu et al., 2016).

Figure 4: A) Anti-bacterial activity of isolated ester derivatives Di-(2-ethylhexyl) phthalate and Dibutyl phthalate: MIC was calculated. The antibiotics Benzylpenicillin and Streptomycin were used as positive control. (B.s = Bacillus subtilis, S.a = Staphylococcus aureus, S.e = Staphylococcus epidermidis, E.c= Escherichia coli, P.a=Pseudomonas aeruginosa, K.p = Klebsiella pneumonia) B) Mosquito larvicidal activity against the Di-(2-ethylhexyl) phthalate. Mortality percentage at particular concentration and 50 percent lethal concentration was calculated. C) Mosquito larvicidal activity against the Dibutyl phthalate. Mortality percentage at particular concentration and 50 percent lethal concentration was calculated. D) Acetylcholinesterase activity against Di-(2-ethylhexyl) phthalate. Percentage inhibition at particular concentration and 50 percent inhibitory concentration was calculated. E) Acetylcholinesterase activity against Dibutyl phthalate. Percentage inhibition at particular concentration and 50 percent inhibitory concentration was calculated.
CONCLUSION

In our present study two phthalate derivatives DEHP and DBP were isolated from Brevibacterium mcbrellneri. From our toxicological and biochemical evaluation studies we infer that these compounds have antibacterial activity and mosquito larvicidal activity with significant acetylcholinesterase inhibition. These phthalate derivatives have also shown cytotoxicity against all cell lines tested by showing cell cycle arrest in G1 phase.

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Conflict of interest

The authors would like to declare no conflict of interest.

Figure 5: Cytotoxicity Assessment: A) MTT proliferation assay. Doxorubicin used as positive control. B) Analysis of DNA content in CHO cells treated with DEHP and DBP in comparison to control. DMSO used as negative control. C) Cell cycle analysis in CHO cells with DMSO. (D-F) Cell cycle analysis in CHO cells with different concentrations of DEHP. (G-I) Cell cycle analysis in CHO cells with different concentrations of DBP.
REFERENCES

Al-Bari MA, Bhuiyan MS, Flores ME, Petrosyan P, Garcia-Varela M, Islam MA. Streptomyces bangladeshensis sp. nov., isolated from soil, which produces bis-(2-ethylhexyl)phthalate. Int J Syst Evol Microbiol. 2005;55:1973-7.

Amade P, Mallea M, Bouaicha N. Isolation, structural identification and biological activity of two metabolites produced by Penicillium olsonii Bainier and Sartory. J Antibiot. 1994;47:201-7.

Chen CY. Biosynthesis of di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) from red alga Bangia atropurpurea. Water Res. 2004;38:1014-8.

Chen L, Huang X. Preparation of an absorbent based on polymeric liquid for the simultaneous extraction of acidic, basic and neutral pollutants. J Chromatogr A. 2016;1466:42-9.

Choi EJ, Kwon HC, Ham J, Yang HO. 6-Hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinedimethanol from a marine bacterium, Brevibacterium sp. KMD 003, associated with marine purple vase sponge. J Antibiot. 2009;62:621-4.

da Silva GN, Trindade FT, Dos Santos F, Gosmann G, AA ES, Gnoatto SC. Larvicidal activity of natural and modified triterpenoids against Aedes aegypti (Diptera: Culicidae). Pest Manag Sci. 2016;72:1883-7.

Esumi H, Lu J, Kurashima Y, Hanaoka T. Antitumor activity of pyrvinium pamoate, 6-(dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1H-pyrrol-3-yl)ethenyl]-1-methyl-qu inolinium pamoate salt, showing preferential cytotoxicity during glucose starvation. Cancer Sci. 2004;95:685-90.

Futamura T, Okabe M, Tamura T, Toda K, Matsunobu T, Park YS. Improvement of production of Kojic acid by a mutant strain Aspergillus oryzae, MK107-39. J Biosci Bioeng. 2001;91:272-6.

Govindarajan M, Kadaikunnan S, Alharbi NS, Benelli G. Single-step biological fabrication of colloidal silver nanoparticles using Hugonia mystax: larvicidal potential against Zika virus, dengue, and malaria vector mosquitoes. Artif Cells Nanomed Biotechnol. 2016 Sep 9;1-9. doi: 10.1080/21691401.2016.1228664. [Epub ahead of print].

Habib MR, Karim MR. Antimicrobial and cytotoxic activity of di-(2-ethylhexyl) phthalate and anhydroso phosphoradiol-3-acetate isolated from Calotropis gigantea (Linn.) flower. Mycobiology. 2009;37:31-6.

Haggis GH, Pipps-Todd B. Freeze-fracture for scanning electron microscopy. J Microsc. 1977;111:193-201.

Heinemeyer G, Sommerfeld C, Springer A, Heiland A, Lindtner O, Greiner M, et al. Estimation of dietary intake of bis(2-ethylhexyl)phthalate (DEHP) by consumption of food in the German population. Int J Hyg Environ Health. 2013;216:472-80.

Holt RJ. Laboratory tests of antifungal drugs. J Clin Pathol. 1975;28:767-74.

Hsu HF, Huang KH, Lu KJ, Chiou SJ, Yen JH, Chang CC, et al. Typhonium blumei extract inhibits proliferation of human lung adenocarcinoma A549 cells via induction of cell cycle arrest and apoptosis. J Ethnopharmacol. 2011;135:492-500.

Kiran GS, Lipton AN, Priyadharshini S, Anitha K, Suarez LE, Arasu MV, et al. Antiadhesive activity of poly-hydroxy butyrate biopolymer from a marine Brevibacterium casei MSI04 against shrimp pathogenic vibrios. Microb Cell Fact. 2014;13:114.

Kumar S, Warikoo R, Wahab N. Larvicidal potential of ethanolic extracts of dried fruits of three species of peppercorns against different instars of an indian strain of dengue fever mosquito, Aedes aegypti L. (Diptera: Culicidae). Parasitol Res. 2010;107:901-7.

Lee DS. Dibutyl phthalate, an alpha-glucosidase inhibitor from Streptomyces melanosporofaciens. J Biosci Bioeng. 2000;89:271-3.

Lee KH, Kim JH, Lim DS, Kim CH. Anti-leukaemic and anti-mutagenic effects of di(2-ethylhexyl)phthalate isolated from Aloe vera Linne. J Pharm Pharmacol. 2000;52:593-8.

Lyu ZQ, Xie X, Ke YB. [Effects of long term and low dose Di- (2-ethylhexyl) phthalate exposure on global genome DNA methylation in HePG2 cells]. Zhonghua lao dong wei sheng zhi ye bing za zhi = Zhonghua laodong weisheng zhiyebing zazhi = Chin J Ind Hyg Occup Dis. 2016;34:346-51.

Majzner K, Wrobel TP, Fedorowicz A, Chlapiacki S, Baransa M. Secondary structure of proteins analyzed ex vivo in vascular wall in diabetic animals using FT-IR spectroscopy. Analyst. 2013;138:7400-10.

Mavar-Manga H, Haddad M, Pieters L, Baccelli C, Penge A, Quetin-Leclercq J. Anti-inflammatory compounds from leaves and root bark of Alchornea cordifolia (Schumach. & Thonn.) Mull. Arg. J Ethnopharmacol. 2008;115:25-9.

McBride ME, Ellner KM, Black HS, Claridge JE, Wolf JE. A new Brevibacterium sp. isolated from infected genital hair of patients with white piedra. J Med Microbiol. 1993;39:255-61.
Morcombe CR, Zilm KW. Chemical shift referencing in MAS solid state NMR. J Magn Reson. 2003;162:479-86.

Nagaveni V, Prabhakar S. Insights into the binding sites of sulforaphane on insulin studied by electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom. 2015;29:1155-64.

Patel N, Garikapati KR, Ramaiah MJ, Polavarapu KK, Bhadra U, Bhadra MP, miR-15a/miR-16 induces mitochondrial dependent apoptosis in breast cancer cells by suppressing oncogene BMI1. Life Sci. 2016;164:60-70.

Peropadre A, Fernández Freire P, Pérez Martín JM, Herrero Ó, Hazen MJ. Endoplasmic reticulum stress as a novel cellular response to di (2-ethylhexyl) phthalate exposure. Toxicol In Vitro. 2015;30:281-7.

Podojil M, Gerber NN. The biosynthesis of 1,6-phenazinediol 5,10-dioxide (Iodinin) by Brevibacterium iodinum. Biochemistry. 1967;6:2701-5.

Prata-Sena M, Ramos AA, Buttachon S, Castro-Carvalho B, Marques P, Dethoup T, et al. Cytotoxic activity of secondary metabolites from marine-derived fungus Neosartorya siamensis in human cancer cells. Phytotther Res. 2016;30:1862-71.

Roux V, Raoul D. Brevibacterium massiliense sp. nov., isolated from a human ankle discharge. Int J Syst Evol Microbiol. 2009;59:1960-4.

Roy RN, Laskar S, Sen SK. Dibutyl phthalate, the bioactive compound produced by Streptomyces albidosflavus 321.2. Microbiol Res. 2006;161:121-6.

Sridhar L, Karthikraj R, Lakshmi VV, Raju NP, Prabhakar S. Rapid screening of N-oxides of chemical warfare agents degradation products by ESI-tandem mass spectrometry. Anal Bioanal Chem. 2014;406:5235-41.

Tao LX, Huang XT, Chen YT, Tang XC, Zhang HY. Acetylcholinesterase-independent protective effects of huperzine A against iron overload-induced oxidative damage and aberrant iron metabolism signaling in rat cortical neurons. Acta Pharmacol Sin. 2016;37:1391-400.

Toth-Soma LT, Gulyas S, Szegletes Z. Functional connection between intracellular and extracellular secretion in species of Euphorbia genus. Acta Biol Hung. 1993;44:433-43.

Xie Y, Liu Z, Zhang G, Mo X, Ding X, Xia L, et al. A rifampicin-resistant (rpoB) mutation in Pseudomonas protegens Pf-5 strain leads to improved antifungal activity and elevated production of secondary metabolites. Res Microbiol. 2016;167:625-9.

Xu H, Shao X, Zhang Z, Zou Y, Chen Y, Han S, et al. Effects of di-n-butyl phthalate and diethyl phthalate on acetylcholinesterase activity and neurotoxicity related gene expression in embryonic zebrafish. Bull Environ Contam Toxicol. 2013;91:635-9.