Isolation, Structure elucidation and Biological Activity of Di-(2-ethylhexyl) phthalate Produced by Penicillium janthinellum 62

Osama H. El-Sayed¹, Mohsen M. S. Asker¹*, Seham M. Shash², Shimaa R. Hamed¹

¹Microbial Biotechnology Department, National Research Centre, 33 Bohouth St. Dokki, Giza, Egypt
²Botany and Microbiology Department, Faculty of Science, Benha University, Egypt

Abstract: On the course of screening program for new bioactive compounds, the present study was carried out on the crude extract of Penicillium janthinellum 62. The crude extract of P. janthinellum 62 was fractionated on silica gel column and Sephadex-LH20, ten active fractions were produced. Fraction 7 showed highest antitumor activity, which then purified using preparative TLC and produced six sub-fractions. Sub-fraction (A) showed highest antitumor activity. Sub-fraction (A) was subjected to many identification instruments including IR, NMR, MS and UV spectroscopy. The obtained results showed that, the suggested chemical structure of the compound may be di-(2-ethylhexyl) phthalate (DEHP) or one of its isomers. DEHP was evaluated for antioxidant, antitumor, antiviral activities and cytotoxicity against two tumor cell lines. Results showed that DEHP has antioxidant activity and the maximum activity was 77.99 % at maximum concentration 12 µg/ml, has antitumor activity using Ehrlich cells and the maximum antitumor activity was 77.29 % at maximum concentration 12 µg/mL. also, DEHP showed cytotoxicity against human breast aden carcinoma cell line (MCF-7) and human alveolar basal epithelial cell line (A-549). In addition, DEHP showed antiviral activity against H1N1 at different concentrations.

Keywords: Biological activity, Di-(2-ethylhexyl) phthalate, Penicillium janthinellum 62.

Introduction

In recent years, numerous metabolites possessing uncommon structures and potent bioactivity have been isolated from strains of bacteria and fungi collected from diverse environments, such as water, soils, plants and sediments¹. Therefore, many pharmaceutical companies and research groups were motivated to start sampling and screening large collections of fungal strains for antibiotics, antioxidants, antiviral anticancer and pharmacologically active agents²⁻⁶. Penicillium is a large anamorphic ascomycetous fungal genus with widespread occurrence in most terrestrial environments. This genus comprises more than 200 described specie and many are common soil inhabitants as well as food borne contaminants or food ingredients used in the preparation of cheese and sausages⁷. Penicillium species produce a much diversified array of active secondary metabolites, including antibacterial, antifungal, immunosuppressant, and cholesterol lowering agents⁸⁻¹¹. Thousands of Penicillium isolates have been screened in bioprospecting programs since the discovery of penicillin and new bioactive metabolites continue to be discovered from these fungi nowadays¹²,¹³ indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industry. Phthalate compounds are a family of man-made chemicals used as plasticizers or solvents in a variety of industrial products. Di-(2-ethylhexyl) phthalate (DEHP) is the most common of the class of phthalate
plasticizers, accounting for an almost 54% market share in 2010. In fact fatty foods such as milk, butter, and meats are found to be the main sources of natural DEHP and other phthalates. DEHP has also been reported as a bioactive compound from terrestrial and marine organisms including *Streptomyces bangladeshiensis*\(^{14}\), *Pseudomonas* sp. PBO\(^{15}\), marine algae\(^{16,17,18}\) and fungi\(^{20,21}\). DEHP is a well known environmental estrogen. Phthalates as a group were considered practically nontoxic\(^{22}\). Animal studies performed to current guidelines have shown a slight skin and eye irritation after administration of DEHP, but DEHP is not corrosive to the skin or eyes. DEHP has not been found to induce skin sensitization in animals\(^{23}\). The primary use of DEHP is as a plasticizer in the production of numerous polymers, particularly polyvinyl chloride (PVC), which accounts for approximately 95% of its consumption\(^{24}\). Flexible PVC is used in many different articles e.g. toys, building material such as flooring, cables, profiles and roofs, as well as in medical products such as blood bags and dialysis equipment. The crude extract of *Penicillium janthinellum*\(^6\) showed interesting antioxidant activity. Investigation of the extract led to the isolation of four active compounds. Their structure was identified by spectroscopic data. Their antitumor, antiviral activities were also evaluated.

**Materials and Methods**

**Collection of samples**

Soil samples were collected and isolation of fungi by dilution plate method was used to determine soil fungi as described by Johnson et al.\(^{25}\).

**Taxonomic study of fungal strain**

Strain No. 62 has been isolated from Egyptian environment (from soil) was studied using the method described by PITT\(^{26}\). The cultural characteristics on Czapek-Dox medium\(^ {27}\) were described after 7 days of incubation at 28°C. The morphological characteristics of the strain were observed under light microscopy. Strain No. 62 was kept in Czapek-Dox medium at 4°C.

**Fermentation and working up**

The well grown single colonies of *P. janthinellum* 62 were inoculated in subculture agar slants containing malt extract medium (g/L): malt extract 30; peptone 5; agar 20; at pH 5.5 for 7 days at 30°C. The obtained grown agar slants were served to inoculate 500 mL Erlenmeyer flasks, each containing 100 mL of GYMP medium (g/L): malt extract 3; yeast extract 3; peptone 5; glucose 10, at pH 6.0 at 28°C. The culture media was in turn applied to cultivation on a rotary shaker at 5 days. After harvesting, the resulting fungal biomass, including the medium, was homogenized in a blender (16000 rpm), and filtered during a filter press. The filtrate was extracted using ethyl acetate as described by Serizawa et al.\(^ {28}\) and collected aqueous ethyl acetate was concentrated in-vacuo. According to TLC monitoring, ethyl acetate extracts of mycelium and supernatant showed high similarity and were combined and followed by concentration in-vacuo to afford 9.0 g as brown oily crude extract. The obtained unique black organic extracts were applied to biological screening against Ehrlich Accites Carcinoma cells (EACC).

**Isolation of the active constituents**

The obtained extract was applied to column chromatography on silica gel G254 eluted by CH\(_2\)Cl\(_2\)-MeOH (99.5: 0.5, v/v) gradient and monitored by TLC using n-hexane: ethyl acetate (9: 1, v/v) as solvent system to afford four fractions. The compounds were detected from their UV absorbance at 254 and 366 nm by spraying the TLC plates with Anisaldehyde/H\(_2\)SO\(_4\) reagent give different color reactions with many structural elements\(^{29,30}\). Every individual fraction was loaded on the top of the Sephadex LH-20 column and CH\(_2\)Cl\(_2\)-MeOH (60:40, v/v) was the mobile phase used in this study. The obtained fractions were applied to antioxidant activity.

**Spectral measurements**

To elucidate the chemical structures of promising active compound, they was analyzed using infrared (IR), mass and nuclear magnetic resonance (NMR) spectroscopy. An IR spectrum of pure compound was performed with KBr pellets, 2.0 mg sample and 200 mg KBr using the FTIR-UNIT Bruker Vector 22 Spectrophotometer. NMR (\(^1\)H and \(^13\)C NMR) were obtained on JEOL-ECA 500MHz spectrometer using TMS as internal standard, the chemical shifts were expressed in d (ppm) and coupling constants J in Hz. Mass spectra were recorded on thermo scientific-trace GC Ultra, USA. Coupled with single quadrupole MS, temperature (0-
300 °C) and UV spectroscopy were recorded on a Shimadzu UV-2401 PC double beam spectrophotometer using 1.0 cm quartz cells according to Paterson and Kemmelmeier31. These experiments were carried out in central services laboratory at National Research Centre, Egypt

Biological Activity

Antioxidant activity

The free radical scavenging activity (RSA) of the isolated active secondary metabolite was assessed by the decolouration of chloroform solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical (violet color) according to Brand-Williams et al.32. The scavenging activity of free radical by active compound was evaluated spectrophotometrically at 517 nm. The experiment was carried out in triplicate and averaged. The scavenging activity was calculated as follows:

Scavenging ability (%) = \( \frac{A_{517 \text{ of control}} - A_{517 \text{ of sample}}}{A_{517 \text{ of control}}} \times 100 \).

Antiviral assays

The antiviral activity of the compounds was determined against A/Puerto Rico/8/1934 (PR8) H1N1 virus. Stock solutions of the test compounds were prepared in DMSO. Cells grown to confluence in 96-well plates were infected with 100 µL of 100 TCID50 viruses. After an adsorption period of 1 h at 37°C, virus was removed and serial dilutions of the compounds were added. Maintenance DMEM with 2% FBS was added (100µl/well). The cultures were further incubated at 37°C for 3 days, until complete CPE was observed in the infected and untreated virus control. The determination of the anti-H1N1 virus activity of the compounds was based on virus-induced cytopathogenicity of H1N1 infected MDCK cells, measured at day 4 post virus infection by the MTT colorimetric method33,34. An absorbance of formazan was detected by a multi-well plate reader at 540 nm with 620 nm reference wavelength. The results were expressed as the 50% effective concentration (EC50). The 50% effective antiviral concentration (EC50) was defined as the compound concentration required for protecting 50% of the virus-infected cells against viral cytopathogenicity.

Assay of the antitumor activity against different cell lines

Cytotoxicity against the 2 tumor cell lines, Human breast Aden carcinoma cell line (MCF-7) and human alveolar basal epithelial cell line (A-549), which were purchased from ATCC, USA, were used to evaluate the cytotoxic effect of the tested samples using MTT Cell Viability Assay according to Hansen et al.,35. Cells (0.5×10⁵ cells/well), in serum-free media, were plated in a flat bottom 96-well microplate, and treated with 20 µL of different concentrations of the tested sample for 48 h at 37° C, in a humidified 5% CO₂ atmosphere. After incubation, media were removed and 40 µl MTT solution/well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 µl of acidified isopropanol/well and plate was shaken at room temperature, followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by <100% relative viability. Percentage of relative viability was calculated using the following equation:

\[
\text{Percentage of relative viability} = \left( \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \right) \times 100
\]

Then the half maximal inhibitory concentration (IC50) was calculated from the equation of the dose response curve.

Results

Cultural and morphological characteristics

The fungal colony characteristically formed smooth, thin-walled stipes. The penicilli were biverticillate, the metulae were mostly divergent but sometimes compact and parallel. The phialides were ampulliform; the conidia were most often subglobose to broadly ellipsoidal, sometimes pyriform with apiculate ends with smooth to finely rough end walls. Colonies on malt agar spreading broadly, thin but of looser texture than on Czapek, heavier sporing, usually in gray to glaucous gray shades, consistently lacking the colored mycelium characterizing the same strains on Czapek and steep agar; exudate not produced; reverse dull yellow-brown, never in red-purple shades; penicilli commonly larger than on Czapek but essentially similar in pattern. These
characteristics indicate that strain No. 62 is *Penicillium janthinellum* 62 Biourge based on the description by PITI.26.

**Extraction and Isolation of DEHP**

The fungus *P. janthinellum* 62 was cultivated at 30°C for 7 days in 100 Erlenmeyer flasks (1 L). The fungal biomass, including the medium, was homogenized in a blender (16000 rpm), and the mixture was extracted with ethyl acetate. After evaporation of the organic phase 9 g of brown oily extract was obtained. The extract was fractionated by silica gel column with a CH₂Cl₂-MeOH (99.5:0.5, v/v) to yield four fractions. Each single fraction was fractionated on a Sephadex LH-20 column, with CH₂Cl₂-MeOH (60:40, v/v). Fraction 1 gave (F1, F2 and F3), Fraction 2 gave (F4, F5 and F6), Fraction 3 gave (F7 and F8) and Fraction 4 gave (F9 and F10). All ten fractions were screened using Ehrlich cells (data not shown). The results revealed that fraction 7 has the best antitumor activity; Fraction 7 was separated into six purified sub-fractions (A, B, C, D, E and F) with n-hexane-ethyl acetate (9:1 v/v) using preparative TLC. These sub-fractions were screened using Ehrlich cells Figure (1).

![Figure 1. Effect of different purified sub-fractions from Fr. 7 of *P. janthinellum* 62 extract on EACC.](image)

**Structure elucidation**

Active sub-fraction A isolated from ethyl acetate extract of *P. janthinellum* 62 was obtained as light yellow oily compound insoluble in water while soluble in ethanol, hexane, ethyl acetate, chloroform and most organic solvents. The isolated compound gave positive reaction (purple color) with concentrated H₂SO₄ but not with ninhydrin reagent. It appeared as a single spot on the TLC Merck Kieselgel 60 F₂54 (Rf: 0.62 with solvent system n-hexane: ethyl acetate 9:1) under long wave length UV light. The molecular weight was established by EI-MS Figure (2) as the following EI-MS: m/z (%) =391.4, [M'] 279.2, 167.1 (C₈H₇O₄), 149 (C₈H₅O₃), 113.1 (C₈H₁₇), 83.1, 71.1, 57.1. The presence of a phthalate was inferred from the EI-MS peak at m/z 167.1 (48) and m/z 149 (100). UV spectra (Ethanol): λ_max= 246.2, 273.4 nm as shown in Figure (3). The IR spectrum revealed a carbonyl band observed at 1653 cm⁻¹, strong C-O band at 1023 cm⁻¹, C-H band at 2917 cm⁻¹, methyl bending vibration band in the range 1412 -1316 cm⁻¹. The ¹H NMR spectrum and ¹³CNMR spectrum were obtained at 500 MHz in CDCl₃ solution. The aromatic signal at 7.25 ppm on ¹H NMR has reasonable coupling constants for protons at the ortho-substituted ring. The ¹³CNMR spectrum confirming the symmetry of molecule as shown in Table (1) and Figure (4).The obtained data, UV, IR, ¹³CNMR, ¹H NMR and MS suggested that the isolated compound may be Di (2-ethylhexyl) phthalate (DEHP).

![Figure 2. EI-MS spectrum of purified sub-fraction A](image)
Figure 3. UV spectrum of purified sub-fraction A

Table 1: $^{13}$CNMR and $^1$HNMR spectrum data

<table>
<thead>
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<th>Code</th>
<th>$^1$HNMR δ, ppm</th>
<th>$^{13}$CNMR δ, ppm</th>
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<tr>
<td>C</td>
<td>4.16</td>
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</tr>
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<td>D</td>
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<td>G</td>
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Figure 4. The suggested structure of purified sub-fraction A

**Biological evaluation of DEHP**

The total antioxidant activity of DEHP was increased by increasing the concentrations of DEHP from 2.0 mg/mL to 12.0 mg/mL and the maximum antioxidant activity was 77.99 % at the maximum concentration of 12 mg/mL as shown in Figure (5). The IC$_{50}$ value against DPPH radical found about 2.0 mg/mL for DEHP after 90 min.

Figure 5. Scavenging effect of DEHP during DPPH test and measured by changes in absorbance at 517 nm.
The inhibition ratio (percent of dead EACC) increased by increasing the concentrations of DEHP from 2.0 to 12.0 mg/mL as shown in Figure (6).

![Figure 6. Antitumor activity using EACC of DEHP at different concentration](image)

The effect of the DEHP on the proliferation of A-549 cells and MCF-7 cells were studied after 48 h of incubation. The treatment of MCF-7 cells with DEHP was more cytotoxic-sensitive than treatment of A-549 as shown in Figure (7). The calculated IC$_{50}$ for cell line MCF-7 was 243 µg/mL for DEHP, while the other cell line A-549 showed a weak cytotoxic level as concluded from the high calculated IC$_{50}$ values, indicating low antitumor affinity to be 370 µg/mL for DEHP.

![Figure 7. Cytotoxic effect of DEHP against A-549 and MCF-7 cells line](image)

The result present in Figure (8) showed that by increasing the concentrations of DEHP and crude extract from 20 µg/mL to 80 µg/mL the cytotoxicity of DEHP and crude extract were increased too. Concentration required to cause a microscopically detectable alteration of normal cell morphology (IC$_{50}$) were 66.1 µg/mL and 29.9 µg/mL for DEHP and crude extract, respectively.

![Figure 8. Cytotoxicity of DEHP and crude extract at different concentration against H1N1](image)
Discussion

The data obtained in the present study, the isolation of di-(2-ethylhexyl) phthalate (DEHP) as a major bioactive compound produced as first time from *P. janthinellum* 62. DEHP is readily absorbed and distributed in the body, but there is no evidence of accumulation. The metabolism of DEHP involves several pathways and yields a variety of metabolites. The major step in the metabolism of DEHP is hydrolysis by lipases to MEHP (mono-(2-ethylhexyl) phthalate) and 2-ethylhexanol. The substance is excreted via the urine, mainly as MEHP-metabolites, but some excretion via bile also occurs in rodents. Acute toxicity studies indicate a low acute toxicity of DEHP. Although there are no adequate acute dermal toxicity data, a low acute dermal toxicity is assumed. Animal studies performed to current guidelines have shown a slight skin and eye irritation after administration of DEHP, but DEHP is not corrosive to the skin or eyes. DEHP has not been found to induce skin sensitization in animals. Microbial derived plasticizers such as DEHP are benign and not only make plastic material flexible but they also offer benefits such as its resistance to migration, evaporation and leaching, stability to light and heat and also have premium biological activities which first recorded as antiviral activity and antioxidant activity in addition to antitumor of DEHP was previously recorded. According to Habib and Karim DEHP showed a significant decrease in viable cell count (p < 0.05), mass gain (due to tumor burden) and elevated the life span of Ehrlich ascites carcinoma cells bearing mice. Altered hematological profiles such as red blood cells, hemoglobin, white blood cells and differential count were reverted to normal levels in DEHP-treated mice. DEHP also brought back altered biochemical parameters (glucose, cholesterol, triglycerides, blood urea) to normal level. Results of this study indicate that DEHP show potent dose dependent antitumor activity against Ehrlich cells in vivo while our studies revealed that DEHP have antitumor activity against Ehrlich cells in vitro also. The DEHP is considered as pro inflammatory agent in other studies. The same compound was isolated from the plant *Aloe vera* and was found to have antileukemic and antimutagenic effects, thus offer environmental friendly plastic for future use.

References


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