Research article

Isolation and characterization of an antibacterial compound Bis-(2-ethylhexyl) phthalate from the fungi *Penicillium digitatum*

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ABSTRACT

Introduction and Aim: Penicillium is a fungal antibiotic derived from *Penicillium notatum*. In this study, we aimed to isolate the antibacterial compound Bis-(2-ethylhexyl) phthalate from the fungi *Penicillium digitatum*, as well as study its antibacterial activity against the the pathogens *Escherichia coli*,Group B *Streptococcus agalactiae*, and *Staphylococcus aureus.*

Materials and Methods: The identification of Bis-(2-ethylhexyl) phthalate-containing secondary metabolite was achieved through bioassay-guided chemical analysis of the crude extract. The structure of the compound was identified from UV spectra, IR, and GC-MS spectral data. The antimicrobial activity of Bis-(2-ethylhexyl) phthalate, isolated from the fungus *Penicillium digitatum,* was determined using standard methods.

Results: UV, IR, and GC-MS spectroscopy confirmed the crude extract of fungi *Penicillium digitatum* to contain the bioactive compounds 1,2-benzenedicarboxylic acid and bis(ethylhexyl) phthalate. The MIC value of the bioactive molecule was also determined to be 32 mg/ml. The *P. digitatum* ethyl acetate extract showed moderate antibacterial activity against the Gram-negative bacterial isolate *Escherichia coli* and high activity against the Gram positive pathogens *Streptococcus agalactiae and Staphylococcus aureus.*

Conclusion: The fungi *Penicillium digitatum* produces the metabolite Bis-(2-ethylhexyl) phthalate, which exhibits antibacterial activity against Gram positive and Gram-negative pathogens and hence could be used in treating infections by these bacterial pathogens.

Keywords. *Penicillium digitatum;* Bis-(2-ethylhexyl) phthalate derivatives; spectroscopic analysis; *Escherichia coli*; *Streptococcus agalactiae*; *Staphylococcus aureus;* antibiotic resistance.

INTRODUCTION

Penicillium derived from *Penicillium notatum*, was the first fungal antibiotic to be discovered (1). Penicillium has been known since 1809. Over the last several decades, natural bioactive molecules have played an important role in the development of new medicines (2). Metabolites with unusual structures and high bioactivity have been identified in recent years from bacteria and fungi found in a wide range of habitats, including water, soil, plants, and sediments. Thus, many pharmaceutical and research organizations have begun collecting and screening extensive collections of fungal strains for pharmacologically active compounds such as antibiotics, antioxidants, antivirals, and cancer treatments (3). Penicillium is a vast genus of anamorphic ascomycetous fungi that are found in almost every terrestrial habitat. There are more than 200 identified species in this genus; many of them are widespread in soil and may be found in food as contaminants or as components in foods like cheese and sausage (4). There is a wide variety of antibacterial, antifungal, immunosuppressant, and cholesterol-lowering medicines produced by *Penicillium* species (5). Bacterial and fungal strains have been isolated from a wide variety of habitats, including water, soils, plants, and sediments1, yielding a plethora of metabolites with novel structures and high bioactivity; In a wider period, between 1981 and 2019, among 164 new antimicrobial drugs approved, 36 were of biological origin, of which 89% were prophylactic agents. In the same period, although fungal diseases were frequently reported, only two antifungal drugs were approved, both of synthetic origin (6). Numerous pathogenic bacteria have demonstrated susceptibility to various natural and synthetic compounds, which serve as potential alternatives to antibiotics owing to their antibacterial properties (7-10). In present study, we aimed to isolated and purification antibacterial compound Bis-(2-ethylhexyl) phthalate from the fungi *Penicillium digitatum* and study their antibacterial activity against select pathogenic Gram-negative as well as Gram-positive bacterial isolates.

MATERIAL AND METHODS

Cultivation of *Penicillium digitatum*

Some citrus fruits showing classic green-mold symptoms were sampled for *Penicillium digitatum* inoculum, which was then grown on a potato dextrose agar (PDA) medium at 27°C for seven days before being stored at 4°C. Hyphae were examined when mature spores were collected by passing spore solutions through a sterilized cotton ball in a funnel after cleaning the PDA surface with autoclaved distilled water.

Identification of *Penicillium digitatum*

Pure mold colonies grown were placed on slides and observed under a microscope. Mycelia and spore structures of *P. digitatum* were examined for their unique morphological characteristics. The morphological characteristic of the fungi captured in the photograph (Fig.1) was identified using a combination of in-depth literature research and expert opinion (11). Distinct morphological features colonies grown at 30 °C on PDA were initially white but become rapidly black during conidial development.

Fig. 1: *Penicillium digitatum* growth on spoiled citrus fruit

UV-Vis, FT-IR and GC-MS spectroscopy

The UV-Visible spectroscopy was used in identifying, characterizing, and quantifying the bioactive compounds in the crude extract of *P.digitatum*. The functional groups in the bioactive compound were determined by Fourier-transform infrared spectroscopy (FTIR) analysis.The Autosampler AS 1300 was used to inject 1 L of *P.digitatum* aqueous extract onto the GC-MS chromatography column (30 m x 0.32 mm x 0.25 m). Initial analysis was performed at 60 °C, and the temperature cycling was scheduled to climb to 240 °C. The temperature was then raised to 290°C over the course of 2 minutes. At a rate of 1mL/min, ultra-pure helium was used as a transport medium. The spectra were obtained in full scan mode with an electron energy of 70 V, allowing for mass ionization in the spectral region of 40-1000 m/z. The compounds were identified by comparing their calculated retention times (RTs) and mass spectra (mas') to a database available at the National Institute of Standards and Technology library (12).

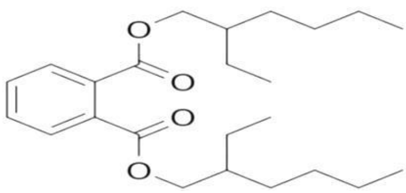

Fig. 2: Structure of Bis-(2-ethylhexyl) phthalate isolated from *P.digitatum*

Antimicrobial susceptibility test

The antibacterial activity of the fungal compound was determined using the disc diffusion assay technique (13) against the Gram positive (Group B *Streptococcus agalactiae*, and *Staphylococcus aureus*) and Gram negative (*Escherichia coli*) isolates. Each bacterial strain was suspended to a turbidity of 0.5 McFarland and plated on Muller-Hinton Agar. Discs containing concentrations of 100 g, 200 g and 400 g of the fungal compound were placed on the agar plates, and incubated at 37°C for 24h. The diameter of the inhibitory zones was measured.

Minimum Inhibition Concentration (MIC)

The MIC of Bis-(2-ethylhexyl) phthalate was determined using the dilution method (14).

RESULTS

The fungi *Penicillium digitatum* was isolated from spoiled citrus fruit in this study. The results of UV-vis spectroscopy showed the crude extract of *P.digitatum* to produce several physiologically active secondary metabolites (Fig.3). The UV-spectrum peak for the bioactive compounds was observed between 205-277 nm (Fig.4).

Fig. 3: UV-vis spectrum of isolated bioactive compound of *Penicillium digitatum*

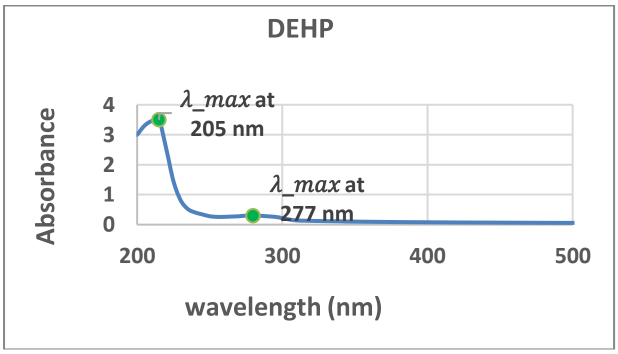

Fig.4: UV spectrum of the Bis-(2-ethylhexyl) phthalate isolated from *P. digitatum*

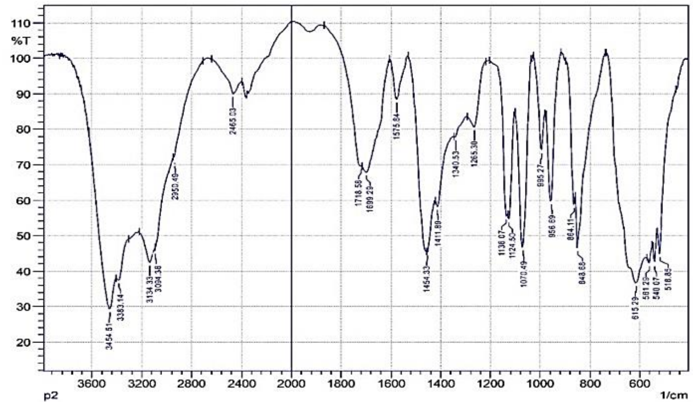

Fig. 5: FTIR spectrum of the *Penicillium digitatum* Bis-(2-ethylhexyl) phthalate bioactive compounds

Table 1: The absorption bands and their structural combination in the FT-IR spectrum

Sl. No	Peak values	Functional groups
1	3454.51, 3383.14	$\mathrm{N-H}$
2	3094.38	$\mathrm{C-H}(sp^2)$
3	2950.48	$\mathrm{C-H}(sp^3)$
4	17118.58	$\mathrm{C=O}$
5	1699.29	$\mathrm{C-H}$
6	1575.84	$\mathrm{C=C}$
7	1454.33	CH_2 bend
8	1340.53	CH3 bend
9	1124.50 - 1136.07	$\mathrm{C-O}$

The FT-IR analysis showed the absorbance spectrum to range between 3446 and 1577 cm^{-1}, which could be assigned to ketone (1722, cm-1), aromatic (3377 and 1577, cm-1), and strong C-O (1261-1070, cm-1) hydroxyl groups (Fig.5). The absorption peak values and their associated functional groups are listed in Table 1.

GC-MS analysis

GC-MS analysis was used to determine the identity of the chemical components in an ethyl acetate extract of *P. digitatum*. The results of the GC-MS analysis showed several major compounds in the extract of the fungi *P. digitatum* (Fig.6), which included Eicosene (4.10%), Tetracosamethyl- cyclododecasiloxane (1.21%), 1-Octadecene (5.67%), Tetracosamethyl-cyclododecasiloxane(5.44%) , (trans)-2-nonadecene(4.54%) , Bis(2-ethylhexyl) phthalate (5.75%), 6-Aza-5,7,12,14-tetrathiapentacene (1.70%), Bis (2- ethylhexyl) sebacate (1.30%), Squalene (53.54%), 4H- Dibenz(de,g}isoquinoline, 5,6,6a,7-tetrahydro-1,2,9,10-tetramethoxy-5-methyl- (1.85%), Ethyl 2-(2-chloroacetamido)-3,3,3-trifluoro-2-(4-fluoroanilino)propionate(3.19%) , 9-Hexadecenoic acid, octadecyl ester (1.75%), 2-(Phenylthio) octanal (2.53%). Cyclotetradecane, 4-isopropyl-1,7,11-trimethyl (4.31%) (Table 2).

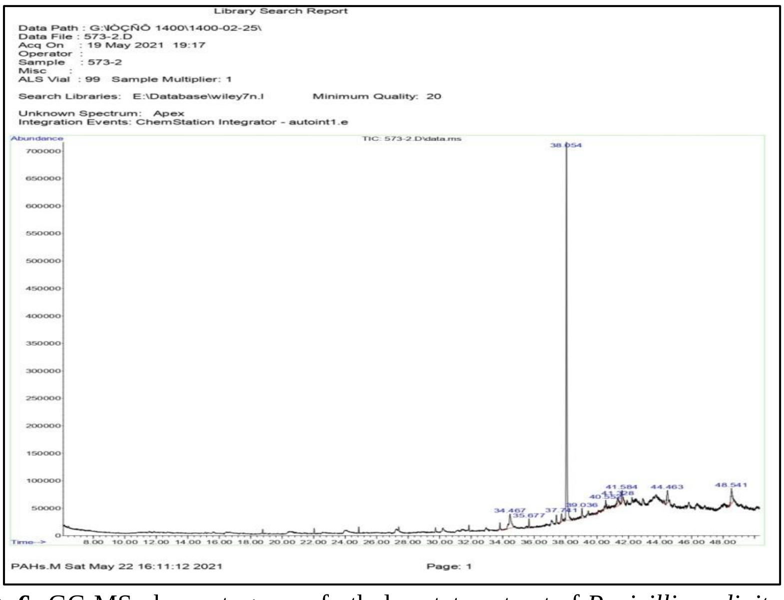

Fig. 6: GC-MS chromatogram of ethyl acetate extract of *Penicillium digitatum*

Table 2: GC-MS chromatogram of compounds in *Penicillium digitatum* extract

No	RT (min)	Area (Ab*s)	Area%	Name	Quality	CAS Number
1	18.763	383987	0.91	1,1,1,3,5,7,9,9,9-Nonamethylpentasiloxane	38	084409-41-6
2	20.47	1734688	4.10	5-Eicosene, (E)-	53	074685-30-6
3	22.026	509667	1.21	Tetracosamethyl-cyclododecasiloxane	47	018919-94-3
4	24.133	2397966	5.67	1-Octadecene	72	000112-88-9
5	27.391	2300938	5.44	Tetracosamethyl-cyclododecasiloxane	35	018919-94-3
6	30.214	1918290	4.54	(trans)-2-nonadecene	76	000000-00-0
7	34.469	2430949	5.75	Bis(2-ethylhexyl) phthalate	80	000117-81-7
8	35.678	717514	1.70	6-Aza-5,7,12,14-tetrathio pentacene	50	000000-00-0
9	37.743	551041	1.30	Bis(2-ethylhexyl) sebacate	38	000122-62-3
10	38.054	22641281	53.54	Squalene	96	007683-64-9
11	40.55	782635	1.85	H-Dibenz(de,g}isoquinoline, 5,6, 6a, 7-tetrahydro-1,2,9,10-tetramethoxy-5-methyl- (CAS)	53	074199-94-3
12	41.328	1348003	3.19	Ethyl 2-(2-chloroacetamido)-3,3,3-trifluoro-2-(4-fluoroaniline)propionate	43	000000-00-0
13	41.582	738772	1.75	9-Hexadecenoic acid, octadecyl ester, (Z)-	41	022393-84-6
14	44.462	1088844	2.57	2-(Phenylthio)octanal	46	000000-00-0
15	48.54	1823225	4.31	Cyclotetradecane, 4-isopropyl-1,7,11-trimethyl-	41	001786-12-5

Table 3: Chemical compounds identified in the ethyl acetate extract of *Penicillium digitatum*

No	Compounds	Chemical formula	Molecular Weight	RT (min)	Area (%)
1	Bis (2-ethylhexyl)phthalate	C_6H_{42}	390,5	34.46	5.75
2	Bis (2-ethylhexyl)Sebacate	$C_{26}H_{50}O_4$	426.6	37.74	1.30
3	5- Eicosene	$C_{20}H_{42}$	282.5	20.47	4.10
4	1-Octadecene	$C_{18}H_{36}$	252.5	24.13	5.67
5	Squalene	$C_{30}H_{50}$	422.8	38.05	53.54
6	2-(phenyl)thio)octanal	$C_{14}H_{16}O_5$	216.3	44.46	2.57
7	Cyclotetradecane trimethyl	$C_{20}H_{40}$	280.5	48.54	4.31

Table 4: The of inhibition zone of Bis-(2-ethylhexyl) phthalate from *Penicillium digitatum* against MDR pathogenic

No	Pathogenic bacterial isolates	Inhibition zone (mm)
1	*Streptococcus agalactiae*	12.00*
2	*Staphylococcus aureus*	14.33*
3	*Escherichia coli*	13.10*

Table 5: The MIC for Penicillin Bis-(2-ethylhexyl) phthalate compound tested against multi-drug *Streptococcus agalactiae*

Sample	Dilution of Bis-(2-ethylhexyl) phthalate Penicillin compound extracted from *Penicillium digitatum* (mg / ml)						
	≥64	**≥32**	≥16	≥8	≥4	≥2	≥1
Streptococcus agalactiae	-	-	+	+	+	+	+

A library search found few of these compounds to be novel. The chemical compounds their chemical formulae and molecular weights are listed in Table 3.

Antibacterial effect of *P. digitatum* extract

The assessment for the antibacterial effectiveness of *P. digitatum* extract showed the extract to significantly inhibit the pathogenic multi-drug resistant bacterial isolates used in testing (Table 5). The MIC of the Bis-(2-ethylhexyl) phthalate isolated from *Penicillium digitatum* was recorded as (≥32 mg / ml) The inhibition zones recorded for *Streptococcus aggalactiae, Staphylococcus aureus* and *E.coli* were 12nm, 14.3nm and 13.1nm respectively (Table 4).

DISCUSSION

In recent years, several new bioactive compounds have been uncovered from microorganisms that have the ability to combat bacterial and fungal pathogens (15). Penicillium derived from *Penicillium notatum* has been shown as a prospective source of bioactive chemicals as they are known to produce a wealth of physiologically active secondary metabolites, essential for helping the fungi to increase their tolerance to different types of environmental stresses and hostile conditions, and consequently their survival (1–3). In this study, UV-vis spectroscopy results for the crude extract of *P.digitatum* showed this fungi to produce several physiologically bioactive compounds in the UV-spectrum range between 205-277 nm. This result was in line with UV-spectrum range observed for secondary metabolites from a *Penicillium spp. and Bacillus cereus* (15,16).

The Bis-(2-ethylhexyl) phthalate purified in this study, showed antibacterial activity against both Gram positive and Gram negative pathogens tested. This is consistent with earlier reports wherein phthalate derivatives from bacteria and fungal origin have been shown to have antibacterial effect on pathogens (17,18). The spectral data analysis compared in prior research (19,20) identified this metabolite as benzene 1, 2-dicarboxylate and Di-(2-ethylhexyl) phthalate (DEHP). The MIC of the Bis-(2-ethylhexyl) phthalate bioactive compound isolated from *Penicillium digitatum* in this study was recorded as (≥32 mg / ml) which was in agreement with an earlier similar work (21). A cytotoxicity assay for the bioactive compound of Bis-(2-ethylhexyl) phthalate on human red blood cells has shown that this compound exerts no cytotoxic effect in the range of 1-32 mg/ml (22). Toxic effects against a wide variety of pathogenic bacteria have been demonstrated for a wide variety of naturally occurring and artificially synthesized substances. The aforementioned compounds have the potential to serve as effective alternatives to antibiotics due to their antibacterial properties (10, 23,24). Hence, we infer that the bioactive molecules produced by *P. digitatum* in this study holds promise in treating multidrug-resistant *Streptococcus agalactiae*, *S. aureus* and *E.coli*. Further studies on the antimicrobial effect of *P. digitatum* secondary metabolites on other bacterial and fungal pathogens is needed.

CONCLUSION

The ethyl acetate extracts of the bioactive compounds of bis(ethylhexyl) phthalate from *Penicillium digitatum* fungi were found to have activities against the test pathogenic bacterial isolated *Streptococcus agalactiae, Staphylococcus aureus* and *Escherichia coli* UV, IR, and GC-MS spectroscopy.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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