



## A New Secondary Metabolite with Antimicrobial, Antioxidant, and Cytotoxic Activities from an Endophytic Fungus, *Gymnoascus thermotolerans*



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A NEW metabolite, a phenolic derivative, 2-tert-butyl-4-(2-methylhex-5-en-2-yl) phenol (**1**), together with three known phthalates (**2-4**), was first isolated from the endophytic fungus *Gymnoascus thermotolerans* which collected from the roots, stems, and leaves of *Euphorbia geniculata* for the first time. The structures were determined by mass spectrometric measurements, 1D and 2D NMR investigations, and mass spectroscopic analyses. The antibacterial, antioxidant, and cytotoxic properties of these metabolites were assessed in vitro. 2-tert-butyl-4-(2-methylhex-5-en-2-yl) phenol (**1**), significantly inhibited the growth of *Staphylococcus aureus* and *Staphylococcus epidermidis* with minimum inhibitory concentration (MIC) values of 9.0, and 10.5  $\mu\text{M}$ , respectively. In contrast, *Escherichia coli*, *Klebsiella pneumoniae*, and *Candida albicans* showed resistance toward the isolated compounds (**1-4**), which displayed low activity against *Candida ciferrii*. However, dibutyl phthalate (**2**) revealed a potent inhibition of spore germination of *Fusarium solani* with (90.8%) at the highest concentration, 2.5mg/mL. Moreover, diisooctyl phthalate (**3**) showed the highest DPPH radical scavenging activity by 72.4% at 500  $\mu\text{g/ml}$  concentration. On the other hand, both dibutyl phthalate (**2**) and 2-(pentyloxy carbonyl) benzoic acid (**4**) exhibited potent cytotoxicity against both MDA-MB-231 cancer cells with ( $\text{IC}_{50}$  0.54, 0.64  $\mu\text{M}$ ) and SKOV 3 cancer cells with ( $\text{IC}_{50}$  0.75, 0.81  $\mu\text{M}$ ), respectively. In addition, the proposed biosynthetic pathways of isolated compounds (**1-4**), as well as their antimicrobial, antioxidant, and cytotoxicity structure-activity relationship, were discussed. Our findings provide new insights into the potential utilization of endophytic fungi as innovative reservoirs for the development of antimicrobial and anticancer therapeutic agents.

**Keywords:** Antimicrobial, Antioxidant, Cytotoxicity, *Gymnoascus thermotolerans*, Phenolic, Phthalates.

### Introduction

Medicinal plants have the ability to produce bioactive secondary metabolites, which aid in the prevention and treatment of diseases (Rebecca et al., 2011; Ahmed et al., 2023; Thapliyal et al., 2023). They are also considered a unique habitat for endophytes (Panche et al., 2016). A

plant endophyte is a group of microorganisms, including fungi and bacteria, that survive inside a plant's internal tissues or organs without showing any outward symptoms of disease in the host plant (Gunatilaka, 2006; Tan & Zou, 2001). The challenge of finding new interesting lead compounds from well-investigated organisms has sparked interest in endophytic fungi as a source of novel bioactive

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Received 20/05/2023; Accepted 04/09/2023

DOI: 10.21608/ejbo.2023.212304.2338

Edited by: Prof. Dr. Ahmed M. Saleh, Faculty of Science at Yanbu, Taibah University, KSA.

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chemicals. Because of their extensive coevolution and genetic recombination, most endophytes have biosynthetic capabilities larger than the host plant (Fernandes et al., 2009). Therefore, Endophytic fungi have been identified as a valuable and unique source of structurally diverse and biologically bioactive secondary metabolites with potential applications in agriculture, medicine, and food industries (Strobel et al., 2004; Gunatilaka, 2006; Verma et al., 2009; El-Debaiky et al., 2023). *Euphorbia geniculata*, known as spurge weed, is an annual plant belonging to the Euphorbiaceae family, represented as herbs, shrubs, and trees (Kumar & Bhowmik, 2010). In Egypt, *E. geniculata* is dispersed as a wild plant in Egypt's Delta farmland, and many secondary metabolites; polyphenols, diterpenes, and triterpenes were isolated from its aerial parts (Rizk et al., 1974; Falodun et al., 2003; Falodun & Agbakwuru, 2004; Falodun et al., 2006; Okoli et al., 2009; James & Friday, 2010; Refahy, 2011; Ghani & Badr, 2020; Ghani et al., 2020). The plant is traditionally used in East Africa to treat gonorrhoea, and migraine, as a purgative, a lactogenic agent, and also has antibacterial and anti-inflammatory properties (Falodun and Agbakwuru, 2004, Falodun et al., 2003, Falodun et al., 2006, James and Friday, 2010, Okoli et al., 2009). The genus *Gymnoascus* (Gymnoascaceae), with *G. reessii* as a generic type species, is considered one of the largest genera in the Onygenales order, composed of 19 species, and commonly isolated from soil, animal's dungs, and marines (Orr et al., 1963; Solé et al., 2002; Okoli et al., 2009). Several bioactive fungal secondary metabolites with anti-malarial, antibacterial, anticancer, and nematocidal properties have been identified from different species of *Gymnoascus* (Clark et al., 2005; Kitchawalit et al., 2014; Liu et al., 2017).

The current study aims to evaluate the secondary metabolites of *G. thermotolerans* as associated endophytic fungi in *E. geniculata* and to assess their bioactivity as part of our ongoing search for potential secondary metabolites from various natural sources. The primary objective of the present investigation is to evaluate the secondary metabolites produced by *G. thermotolerans*, which is recognized as an endophytic fungus associated with *E. geniculata*. Assessing their bioactivity is an integral part of our ongoing quest for potential secondary metabolites derived from diverse natural sources. Specifically, our methodology involved isolating and identifying the thermophilic

endophytic fungus *G. thermotolerans* obtained from *E. geniculata*. Subsequently, the secreted metabolites of *G. thermotolerans* were gathered and subjected to fractionation through column chromatography, resulting in the isolation of pure compounds. These isolated pure compounds were identified through NMR spectroscopy, followed by an evaluation of their antimicrobial, anticancer, and antioxidant properties.

## Materials and Methods

### General procedures

IR spectra were measured with Agilent Technologies Cary 630 FTIR in KBr pellets. NMR spectra were performed on a Bruker Avance III spectrometer (Unity Plus 400MHz). As the internal standard, the solvent residual was used to determine chemical shifts. The mass spectra were recorded on an Agilent 6520 Accurate-Mass Q-TOF LC-MS spectrometer. TLC and PTLC were performed on silica gel 60 GF<sub>254</sub>, and silica gel (200–300 or 300–400 mesh) was used for column chromatography. TLC was used to monitor the fractions, and spots were seen by heating after sprinkling 5% H<sub>2</sub>SO<sub>4</sub> in ethanol. Other substances employed in this research are all of the analytical grades.

### Fungal material

The plant *E. geniculata* was collected from the campus of Aswan University, Aswan governorate, Egypt, in August 2021 and the plant surface was sterilized in 70% (v/v) ethanol for 1min and then in 5% (v/v) sodium hypochlorite solution for 5 min. The sterilized plant was subsequently washed twice with sterilized distilled water (Zhou et al., 2016). Roots stem, and leaves were cut into segments measuring 0.5 to 1cm long, and the resulting specimens were then put directly on a Petri dish that had been sterilized and filled with Potato Dextrose Agar (PDA) medium that had been supplemented with chloramphenicol (100g/mL) to prevent bacteria from growing (Ezeobiora et al., 2021). The three replicated plates were incubated at 45°C for 2–3 weeks. The growing fungi were purified in a new PDA plate. The purified endophytic isolates are kept by transferring onto PDA agar slants containing chloramphenicol at 4°C till further use (Tsuchida et al., 2002). Then these fungi were identified morphologically based on their colonial and hyphal characteristics according to Zhou et al. (2016) and molecularly by rRNA gene sequencing

using the CTAB method. Two fungal primers, ITS1 and ITS4, were used to amplify the partial rDNA gene fragment. Electrophoreses on 1% agarose gel were used to find the PCR amplifications. Korea Solgent Corporation eluted and sequenced these bands. NCBI Blast website was used to analyze sequences (Suarez et al., 2005; Gontia-Mishra et al., 2014).

#### *Fermentation and cultivation*

Inside a sterilized laminar airflow hood and under more precautions the isolated endophytic fungal strain *G. thermotolerans* was incubated with potato dextrose broth (PDB) in a 500mL flask. Then flasks were incubated under shaking conditions (180rpm) for 20 days. The fungal culture received a total of 250mL of ethyl acetate (EtOAc) and was left overnight. Then, the mixture was applied to Ultrasonic for 10min (for cell destruction), followed by Büchner vacuum filtration. The extracted mycelia (cell debris) were discarded away, and the filtrate containing the EtOAc phase and medium (water phase) was collected for further workup. After that, a separator funnel separated the EtOAc phase from the water phase (medium). The residual salts and other polar components were removed by washing the ethyl acetate phase twice with water. Then this phase was collected and evaporated in a vacuum using a rotary evaporator (Xu, 2010). The EtOAc extract was fractionated using a flash column into pure *n*-hexane, *n*-hexane: methylene chloride (1: 1), pure methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>), CH<sub>2</sub>Cl<sub>2</sub>: MeOH (1:1), and pure MeOH fractions. The isolated compounds were extracted from pure methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) and CH<sub>2</sub>Cl<sub>2</sub>: MeOH (1:1) fractions.

#### *Extraction and isolation*

The EtOAc extract (19gm) was separated by silica gel CC using *n*-hexane, CH<sub>2</sub>Cl<sub>2</sub>, and MeOH (gradually) to provide five fractions. Fraction 3 (pure CH<sub>2</sub>Cl<sub>2</sub>) was separated by PTLC and repeated C on silica gel to yield compounds **4** (15mg) and **1** (9.5mg). Fraction 4 (CH<sub>2</sub>Cl<sub>2</sub>/MeOH= 1:1) was separated by repeated CC on silica gel, followed by purification with PTLC to yield compounds **2** (13mg) and **3** (12.5mg).

*2-tert-butyl-4-(2-methylhex-5-en-2-yl) phenol* (Compound **1**): Yellowish powder; IR (KBr)  $\nu = 3443, 2927, 1630, 1571, 1461, 1221 \text{ cm}^{-1}$ . The <sup>1</sup>H-NMR assignment was achieved by <sup>1</sup>H-<sup>1</sup>H correlation spectroscopy COSY, see Table

1; <sup>13</sup>C-NMR (CDCl<sub>3</sub>): the <sup>13</sup>C attributions were achieved by HMQC and HMBC, see Table 1; HRCIMS *m/z* 247.1987 (M+H)<sup>+</sup>, C<sub>17</sub>H<sub>27</sub>O; calcd. 247.1945.

#### *Antimicrobial assay*

##### *Disc diffusion method*

The isolated metabolites were tested *in vitro* for antibacterial and antifungal activities against four pathogenic bacteria: *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae*, and two pathogenic fungi: *C. ciferrii* and *C. albicans*, which had all previously been cultured on nutrient agar medium. The agar disc diffusion method carried out the antibacterial and antifungal assay. It was done by scattering bacteria on top of a nutrient agar medium while using sterilized paper discs saturated with all components dissolved in methylene chloride, incubating the plate overnight at 37°C, and measuring the presence or absence of inhibition zone around the disks (Gupta et al., 2015).

##### *Minimal inhibitory concentration (MIC) assay*

The minimum concentration of isolated compounds (**1-4**) at which no growth of microorganisms occurred (MIC), was determined against both *S. aureus* and *S. epidermidis* strains by using the broth dilution method (Loizzo et al., 2004). Nutrient broth and Sabouraud Dextrose broth (100  $\mu$ l) were distributed into 96- wells plates. Each compound (**1-4**) was added to the first well followed by 10 serial two-fold dilutions. Bacterial and fungal suspensions were added to all wells except the negative control wells. Plates were incubated at 37°C for 48h. The minimum inhibitory concentration was determined by visual examination of the culture turbidity.

##### *Evan blue method*

The isolated compounds were also assessed against *F. solani* by the Evans Blue Staining method which indicated cell death. Spores of *F. solani* were incubated in Potato Dextrose Broth (PDB) at 28°C for five days. The culture was filtrated to remove mycelia. Conidial cells of the fungus were obtained by centrifugation (5000 rpm, 10min). The isolated compounds (**1-4**) with two concentrations (1.25 and 2.5mg/mL) were added to the tubes containing the spores; in addition, control as inoculum only. All the tubes were incubated at 28°C for 24h then the tubes were centrifuged; the culture media were removed and two drops of Evans blue (0.05%) were added for 10min. The hyphae were then cleaned with sterile distilled

water to eliminate any remaining discoloration. The difference in staining in the dead and live cells was observed under a light microscope and by a hemocytometer we calculated the death percentage of the cells (Silva & Menéndez, 2006; Semighini & Harris, 2010).

#### Antioxidant assay

The antioxidant activity of the isolated metabolites (**1-4**) has been determined by DPPH free radical scavenging activity method (Masaki et al., 1997). To check the antioxidant activity through free radical scavenging by the test samples, the change in optical density of DPPH radicals was monitored. Endophytic fungal extract and fractions (1mg/mL) concentration were used. DPPH solution (0.5mmol/L) was prepared in 95% methanol. Methanol was used to dilute the sample extract (0.1mL). The test sample was added to a total of 2mL of DPPH solution (0.5mmol/L), which was then left to incubate at room temperature in complete darkness for 30 minutes. After 30min, the absorbance was measured at 517nm.

#### Cytotoxicity assay

Ovarian Cancer (SKOV-3) and Breast Cancer (MDA-MB-231) cell line were obtained from Nawah Scientific Inc. (Mokatam, Cairo, Egypt). Cells were maintained in RPMI media supplemented with 100mg/mL of streptomycin, 100 units/mL of penicillin, and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO<sub>2</sub> atmosphere at 37°C.

The cytotoxic effect of endophytic fungal extracts (GTE 21) was tested by using SRB assay against human breast Cancer (MDA-MB-231) and Ovarian Cancer (SKOV-3) cell lines using different concentrations of fungal extracts (0.01, 0.1, 1, 10, 50 and 100ug/mL). The surviving fraction and viability were determined (Allam et al., 2018).

#### Statistical analysis

Each experiment was evaluated independently in triplicate. The one-way analysis of variance was applied to the data (ANOVA). Tukey tests revealed significant differences between the control and treatments at level (P 0.05). The MINITAB software's Tukey test was used to analyze differences between means.

## Results and Discussion

From *E. geniculata*, the white fungus was isolated at PDA media at 45°C. Based on the morphological and cultural characteristics viz., we confirmed the fungus identification as *G. thermotolerans* GTE-21 with accession number MW590717 (Zhou et al., 2016).

#### Identification of isolated compounds

In our study, four fungal secondary metabolites (**1-4**) (Fig. 1) were isolated from *G. thermotolerans* that showed variable antimicrobial, antioxidant, and cytotoxic activities. The proposed biosynthetic pathway and structure-activity relationship was discussed. To our knowledge, compound **1** was identified as a new natural metabolite, and compounds (**2-4**) were first reported from the endophytic *G. thermotolerans* which was also isolated for the first time from *E. geniculata* growing in Egypt.

Repetitive chromatographic steps of the EtOAc extract of *Gymnoascus thermotolerans* afforded a new compound **1** and three known phthalates (**2-4**) (Fig. 1).

Compound **1** was obtained as a yellowish powder. The low-resolution mass spectrum exhibited a [M+Na]<sup>+</sup> at *m/z* 269, in accord with the molecular formula C<sub>17</sub>H<sub>26</sub>O. The structure of **1** was determined from careful investigation of the 1D and 2D NMR data. The <sup>1</sup>H-NMR spectrum (Table 1) indicated the presence of two singlet signals at δ 1.31 and 1.36 for the tertiary methyls (H-15, H-16, H-17) and germinal methyls (H-12 and H-13), respectively. The aliphatic methylene protons appeared as two multiplet signals at δ 1.32 and 2.08 for H-8 and H-9, respectively. Moreover, it showed the olefinic methine proton as doublet of doublet at δ 5.82 (*J*= 8, 8 Hz), H-10, while the olefinic methylene protons appeared as doublet of doublet (*J*= 8, 8 Hz) and a multiplet signals at δ 4.96 and 5.40 respectively, which showed a strong correlation in <sup>1</sup>H-<sup>13</sup>C COSY with a carbon signal at δ 114.0, C-11. Furthermore, it showed the aromatic protons (ABX system) as follows: broad singlet at δ 7.38, H-3; doublet of doublet (*J*= 8, 3 Hz) at δ 7.15, H-5; and a doublet (*J*= 8 Hz) at δ 7.56, H-6. The <sup>13</sup>C-NMR data (Table 1) revealed the presence of 17 carbon atoms, and their multiplicity (by DEPT analysis) confirmed the number of atoms of the formula given above. The carbon atoms were assigned as five methyl carbon atoms at δ 31.0 (tert. methyls C-15, C-16, C-17) and 30.1 for the germinal methyls (C-12

and C-13), respectively; three methylene carbon atoms at  $\delta$  23.2, 32.1, 114.0 for C-8, C-9, and C-11, respectively, four methine carbon atoms at  $\delta$  124.0, 124.2, 119.0, 138.5 for C-3, C-5, C-6, and C-10, respectively and five quaternary carbon atoms at  $\delta$  148.2, 147.3, 138.0, 35.6 and 35.4 for C-1, C-2, C-4, C-7, and C-14 respectively. Moreover, all proton and carbon signals were determined by  $^1\text{H}$ - $^1\text{H}$  COSY, HMQC, and HMBC (Table 1, Suppl. Figs. S1, and S2). Confirmation of the structure of **1** was given by the results of the 2D-long range heteronuclear correlation (HMBC) analysis (Table 1). The most correlations were observed between H-3 ( $\delta=7.38$ , bs) with C-1 ( $\delta=148.2$ ) and C-14 ( $\delta=35.5$ ); H-6 ( $\delta=7.56$ , d) with C-1 ( $\delta=148.2$ ); tert. methyls (H-15, 16, 17 ( $\delta=1.31$ , s) with C-14 ( $\delta=35.5$ ); germinal methyls (H-12, H-13 ( $\delta=1.36$ , s) with C-4 ( $\delta=138.0$ ). Therefore, compound **1** was assigned as a new natural product, a 2-tert-butyl-4-(2-methylhex-5-en-2-yl) phenol.

The structure of the known phthalates: diisobutyl phthalate (DIBP) **2**, diisooctyl phthalate (DIOP) **3**, and mono-*n*-pentyl phthalate (MPeP) **4** has been deduced by comparison of their spectral data with those in literature (Debbab et al., 2010; Tian et al., 2016; Wang et al., 2019; Raj, 2020; Haung et al., 2021).

#### Antimicrobial activity

A large number of endophytic metabolites have been shown to possess potential antimicrobial

activity. In the present study, the isolated secondary metabolites (**1-4**) were assessed for antimicrobial activity against two strains of gram-positive bacteria, *S. aureus* & *S. epidermidis*, two-gram negative; *Escherichia coli* & *Klebsiella pneumoniae*, and three strains of fungi; *C. ciferrii*, *C. albicans*, and *F. solani*. The results showed that the tested compounds inhibited the growth of both stains with inhibition zone ranging from 0-7.33mm (Figs. 2, 3). The highest antibacterial activity against *S. aureus* and *S. epidermidis* was observed with compound **1**, showing an inhibition zone (7.3, 6.0mm), followed by **4** (6.3, 6.6mm) and **2** (4.3, 6.0mm), respectively. In contrast, compound **3** showed the lowest activity against both *S. aureus* and *S. epidermidis*, with an inhibition zone of 2.6mm against *S. aureus*, and has no activity against *S. epidermidis* (Figs. 2, 3). Both *E. coli* and *K. pneumoniae* showed resistance toward the tested compounds **1-4** (Figs. 2, 3).

Both *S. aureus* and *S. epidermidis* were sensitive to **1** (MIC 9.0, 10.5  $\mu\text{L mL}^{-1}$ ) and **4** (MIC 10.5, 10.0  $\mu\text{L mL}^{-1}$ ) more than **2** (MIC 15.7, 10.5  $\mu\text{L mL}^{-1}$ ) respectively. However, *S. aureus* showed weak sensitivity against **3** (MIC 25.2  $\mu\text{L mL}^{-1}$ ), while *S. epidermidis* showed resistance toward **2**. On the other hand, *C. albicans* displayed a resistance against compounds **1-4** which showed weak antifungal activity against *C. ciferrii* with an inhibition zone ranging from 1-4.3mm (Figs. 2, 3).

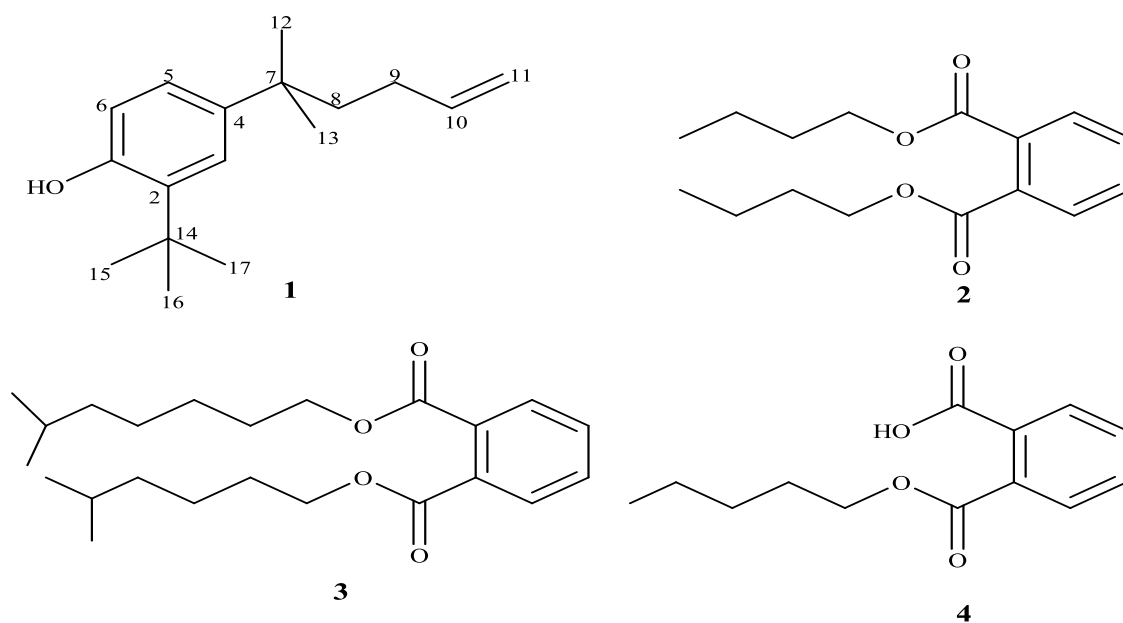


Fig. 1. Isolated secondary metabolites (**1-4**) from *G. thermotolerans*.

TABLE 1. <sup>1</sup>H-NMR (600MHz, CDCl<sub>3</sub>), <sup>13</sup>C- NMR (125MHz, CDCl<sub>3</sub>) spectral data of compound (1)

No	Chemical shift ( $\delta_H$ ) in ppm	Chemical shift ( $\delta_C$ ) in ppm	<sup>1</sup> H- <sup>1</sup> H COSY	HMBC
1	-	148.2, s		
2	-	147.3, s		
3	7.38, brs	124.0, d	H-5	C-1, C-14
4	-	138.0, s		
5	7.15, dd, (J= 8, 3 Hz)	124.2, d	H-3, H-6	
6	7.56, d, (J= 8 Hz)	119.0, d	H-5	C-1
7	-	35.6, s		
8	1.32, m	23.2, t	H-9	
9	2.08, m	32.1, t	H-8, H-10	
10	5.82, dd, (J= 8, 8 Hz)	138.5, d	H-11, H-9	
11	4.96, dd, (J= 8, 8) 5.40, m	114.0, t	H-10	
12	1.36, s	30.1, q		C-4
13	1.36, s	30.1, q		C-4
14	-	35.4, s		
15	1.31, s	31.0, q		C-14
16	1.31, s	31.0, q		C-14
17	1.31, s	31.0, q		C-14

s: singlet; brs: broad singlet; d: doublet; t: triplet; q: quartet; m: multiplet

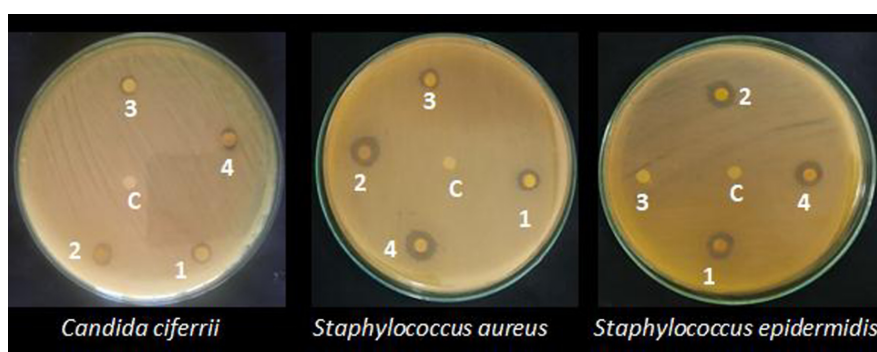


Fig. 2. Antimicrobial activities of compounds (1-4) from *G. thermotolerans*

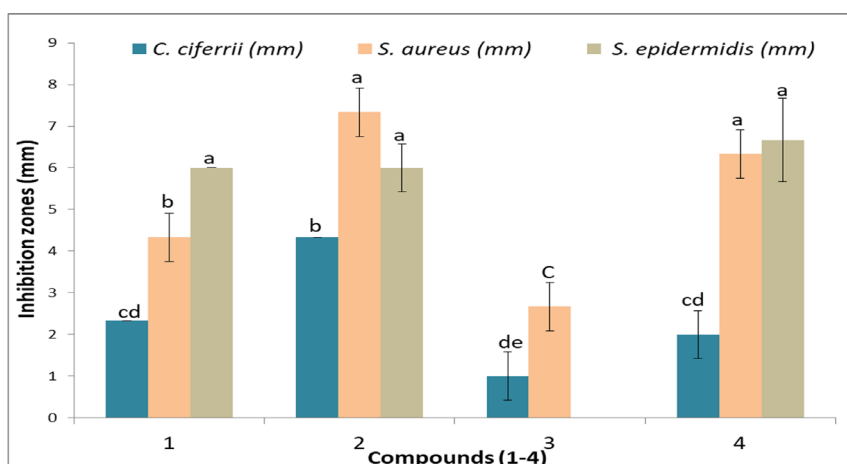


Fig. 3. Inhibition zones in mm of compounds (1-4) against *C. ciferrii*, *S. aureus*, and *S. epidermidis* [Letters (a to e) indicate significant differences  $P < 0.05$  (ANOVA after Tukeys test analysis)]

Additionally, compounds (**1-4**) were also evaluated for *in vitro* *F. solani* spore germination test by Evan blue staining method at five different concentrations (0.25, 0.5, 1, 1.25, and 2.5mg/mL) of each compound (Figs. 4-6). The results revealed that the maximum inhibition of spore germination of *F. solani* with compound **2** (90.8%), followed by **4** (56.8%), **3** (31.8%), and **1** (30.4%) at the highest concentration, 2.5mg/mL (Figs. 4, 6). However, the tested compounds showed relatively low suppression of spore germination (< 26%) detected at 1.25 mg/ml concentration, and all tested compounds have no inhibition of spore germination at the lower concentrations (0.25, 0.5, and 1mg/mL), indicating the inhibition of spore germination of *F. solani* increased in a dose-dependent manner (Figs. 4, 6). Interestingly, **2** showed the lowest inhibition percentage at 1.25 mg/ml toward *F. solani*, and also the other tested compounds displayed a low inhibition percentage at 1.25mg/mL, which increased at 2.5mg/mL, indicating that the antifungal activity of tested compounds against *F. solani* increased in a dose-dependent manner (Figs. 5-7).

#### Antimicrobial and Structure-activity Relationship

In light of our results, we can explain the antimicrobial structure relationship, as it was found that *S. aureus* and *S. epidermidis* were sensitive to **1** as a phenolic compound more than **2-4**, indicating the phenolic moiety of **1** was favorable to enhance its antibacterial activity more than the phthalate esters of **2-4**

(Figs. 2, 3). Interestingly, **4** was more active than **2** toward *S. aureus* and *S. epidermidis*, demonstrating that both organisms are susceptible to the pentyl alkyl group of ester beside the free carboxylic group of **4** more than the two butyl groups of diesters in **2** (Figs. 2, 3). However, *S. aureus* showed less sensitivity toward the two isooctyl moieties as a long alkyl group of phthalate diester of **3**, while both *S. epidermidis* and *C. ciferrii* were nearly resistant against **3** and showed nearly the same sensitivity towards **2** and **4**, indicating the presence of diisooctyl moieties as high-length alkyl groups in **3** lead to abolish its activity against *S. epidermidis* and also highly reducing its efficacy against *C. ciferrii* (Figs. 2, 3).

On the other hand, the phytopathogen *F. solani* was high-sensitive toward the free carboxylic group with a pentyl moiety of monoester of **4** (21.0%) more than the two butyl group of diester **2** (9.9%) at in low concentration (Figs. 4, 6). In contrast, *F. solani* showed the highest sensitivity toward the two butyl groups of diester **2** (90.8%), more than the free carboxylic group with the pentyl moiety of **4** (56.8%) at their concentration of 2.5mg/mL, indicating both the chemical structure of drugs and their concentrations have a significant impact in their antifungal activity against *F. solani* (Figs. 5, 6). The obtained results may be explained by the length of the alkyl group, which is related to the lipophilicity of phthalate esters. Thus, they may be necessary for their antimicrobial activity (Masaki et al., 1997).

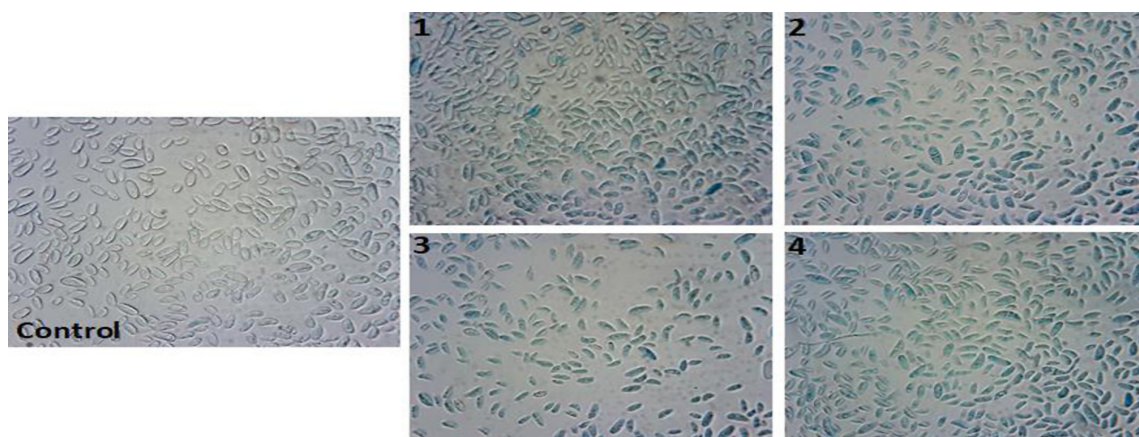


Fig. 4. Effect of compounds (**1-4**) in 1.25mg/mL against *F. solani* by Evan blue method

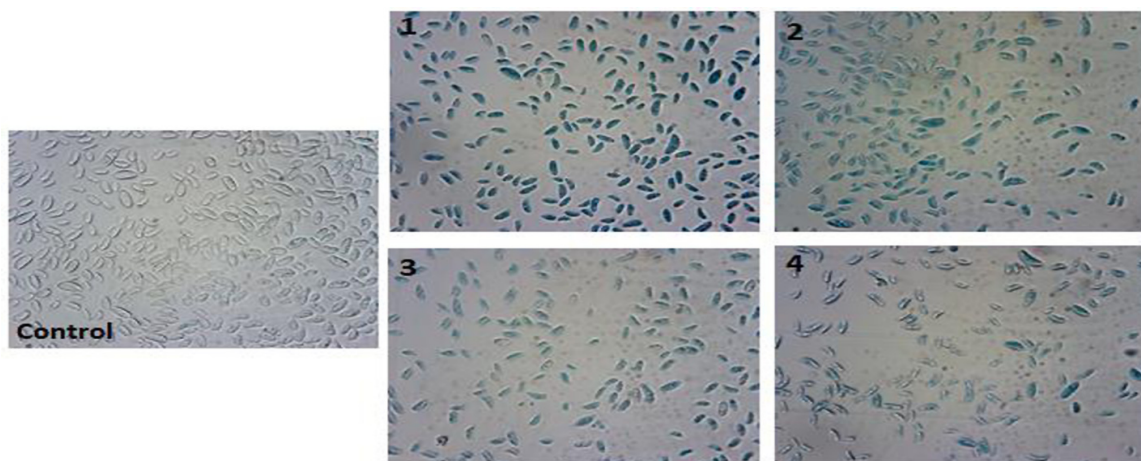


Fig. 5. Effect of compounds (1-4) in 2.5mg/mL against *F. solani* by Evan blue method

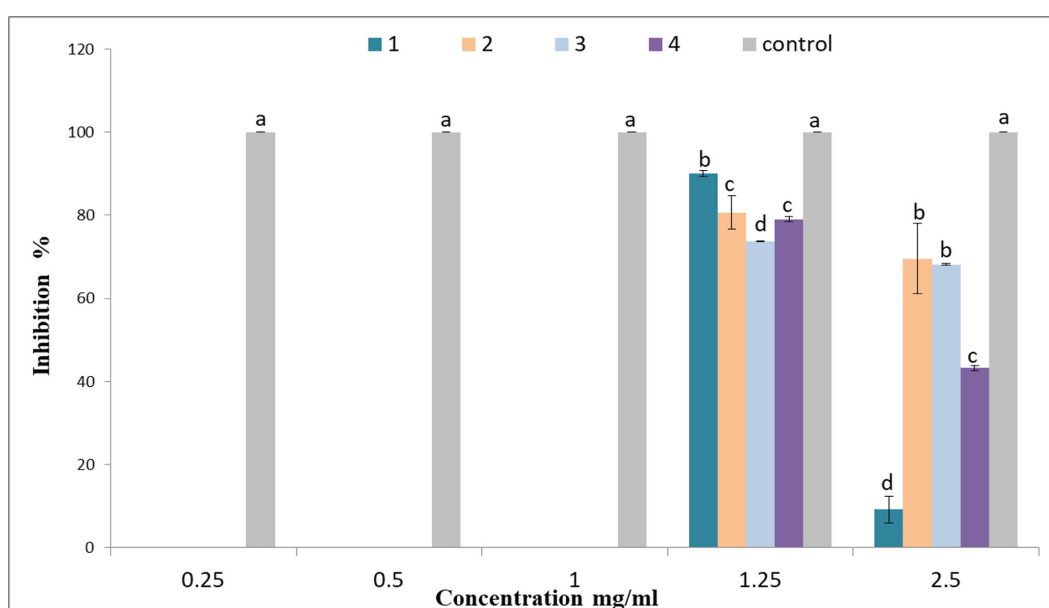


Fig. 6. Effect of compounds (1-4) on spore germination of *F. solani* [Letters (a to d) indicate significant differences  $p < 0.05$  (ANOVA after Tukeys test analysis)]

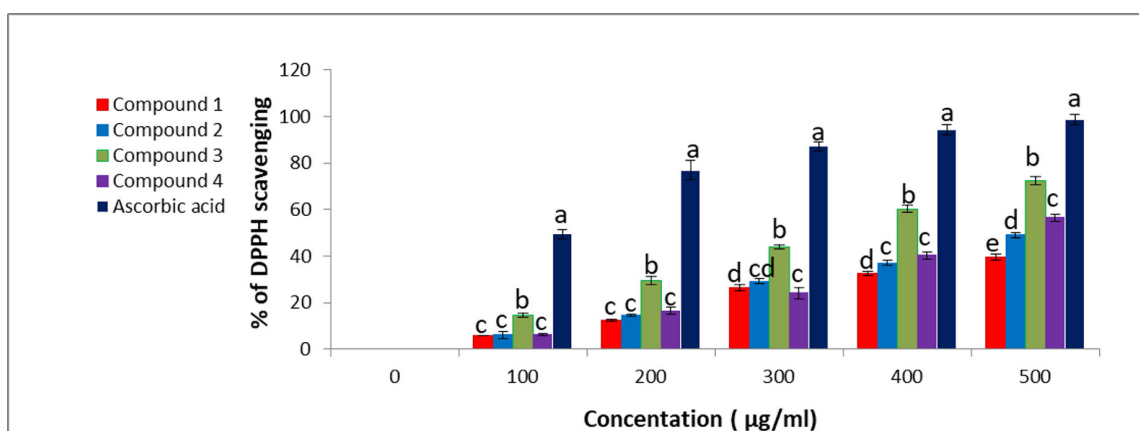


Fig. 7. DPPH free radical scavenging activity of the isolated compounds (1-4) [Letters (a to e) indicate significant differences  $p < 0.05$  (ANOVA after Tukeys test analysis)]



#### *Antioxidant activity*

Natural antioxidants have attracted a lot of attention as cancer chemopreventive agents since reactive oxygen radicals play a significant role in carcinogenesis and other human diseases. Although different assays have been reported to estimate free radical scavenging activity, DPPH is a standard method (Pal et al., 2009). The ability of testing samples as antioxidant activity was evaluated by measuring the change in absorbance caused by reducing DPPH. According to the principle, the antioxidant potential of the examined samples can be determined by their ability to scavenge DPPH radicals. The dose-response curves (Fig. 7) exhibit the DPPH free radical scavenging activity of the isolated compounds (**1-4**) at different concentrations (100-500 µg/mL), compared to ascorbic acid as a standard compound. The isolated compounds were observed to have varying degrees of free radical scavenging ability, which increased dose-dependently. The inhibition percentage of DPPH radical formation ranged from 39.5 to 72.4 % at the highest tested dose (500 µg/ml) and from 2.8 % to 17.1 % at the lowest tested dose (100 µg/mL) (Fig. 4). The obtained results showed that compound **3** had DPPH scavenging activity by 72.4% more than **4** (56.4 %), **2** (48.5%), and **1** (39.5%) at 500 µg/mL concentration.

#### *Antioxidant and structure relationship*

The antioxidant activity of isolated compounds can be correlated with their chemical structures. The presence of two long alkyl chains (diisooctyl) of diester **3** demonstrated the highest free radical scavenging ability compared to **4**, **2**, and **1** (Figs. 1, 7). However, compound **4** comes in the second order regarding its antioxidant activity due to its possessing free carboxylic and pentyl alkyl monoester group in its structure (Figs. 1, 7). While compound **2** comes in the third order because it contains two shorter alkyl (dibutyl) groups of diesters compared to **3** and **4** (Figs. 1, 7). In contrast, compound **1** as a simple phenolic one showed less accessible radical scavenging ability than **2**, **3**, and **4**, classified as phthalate esters. Hence, we can conclude that the length of the alkyl group of phthalate esters plays an essential role in increasing their free radical scavenging ability, as the highest length of the two alkyl groups (isooctyl) of phthalate was found in **3**, which showed the highest activity (**3** vs. **2&4**). On the contrary, it was found that shortening the alkyl group of phthalates harmed their antioxidant activity, as the presence of pentyl and ortho-carboxylic group in **4** instead

of two ortho isooctyl groups in **3** significantly reduced its activity compared to **3** (**4** vs. **3**). It was also found that the shortest alkyl group (butyl) of phthalate diesters was in **2**. As a result, it was less active than **3** and **4** (**2** vs. **3** & **4**). Although the isolated compounds (**1-4**) are aromatic compounds containing one aromatic ring, the difference in their functional groups leads to the variance of their antioxidant activity, and since compound **1**, as a simple phenolic one, was less effective than phthalate esters **2-3** in terms of antioxidant activity (**1** vs. **2-4**).

#### *Cytotoxic activity*

Cancer is a public health challenge and one of the leading causes of mortality worldwide (Denizot & Lang 1986). Breast and ovarian cancer are two of the most frequent cancers in women worldwide. Breast cancer is the most commonly diagnosed malignancy in women, while ovarian cancer is the eighth most prevalent cancer in terms of incidence and mortality (Sung et al., 2021). Several natural products, either extracts or secondary metabolites, have been found to have cytotoxic potential (Cragg et al., 1997; Harvey et al., 2015; Romano & Tatonetti, 2019). Therefore, the isolated compounds **1-4** in the present study were evaluated *in vitro* against two cancer cell lines; breast cancer cells (MDA-MB-231) and ovarian cancer cells (SKOV 3). The assessment of each sample was done at five concentrations (0.01-100 µM) using Sulforhodamine B (SRB) assay (Skehan et al., 1990). The results of our study showed that compounds (**1-4**) inhibited the proliferation of MDA-MB-231 cancer cells (Figs. 8, 10) and SKOV 3 cancer cells (Figs. 9, 10) with various values, which increased in a dose-dependent manner. The cytotoxicity of the tested compounds toward MDA-MB-231 cancer cells was observed with IC<sub>50</sub> in a range (0.54 -1.12 µM) (Fig. 8). The highest cytotoxicity was observed with **2** against MDA-MB-231 cancer cells showing an IC<sub>50</sub> of 0.54, followed by **4**, **1**, and **3** with respective IC<sub>50</sub> values of 0.62, 1.12, and 70.6 µM. On the other hand, the isolated compound **1-4** showed variable cytotoxic activity against SKOV 3 cancer cells with IC<sub>50</sub> ranging (0.75 - >100 µM) (Fig. 9). Compound **2** should also be the strongest cytotoxicity toward SKOV 3 cancer cells with IC<sub>50</sub> 0.75 µM, followed by **4** (IC<sub>50</sub> 0.81 µM) and **1** (IC<sub>50</sub> 1.26 µM), however, the SKOV 3 cancer cells were resistant to **3** with IC<sub>50</sub> (>100 µM) (Fig. 9).

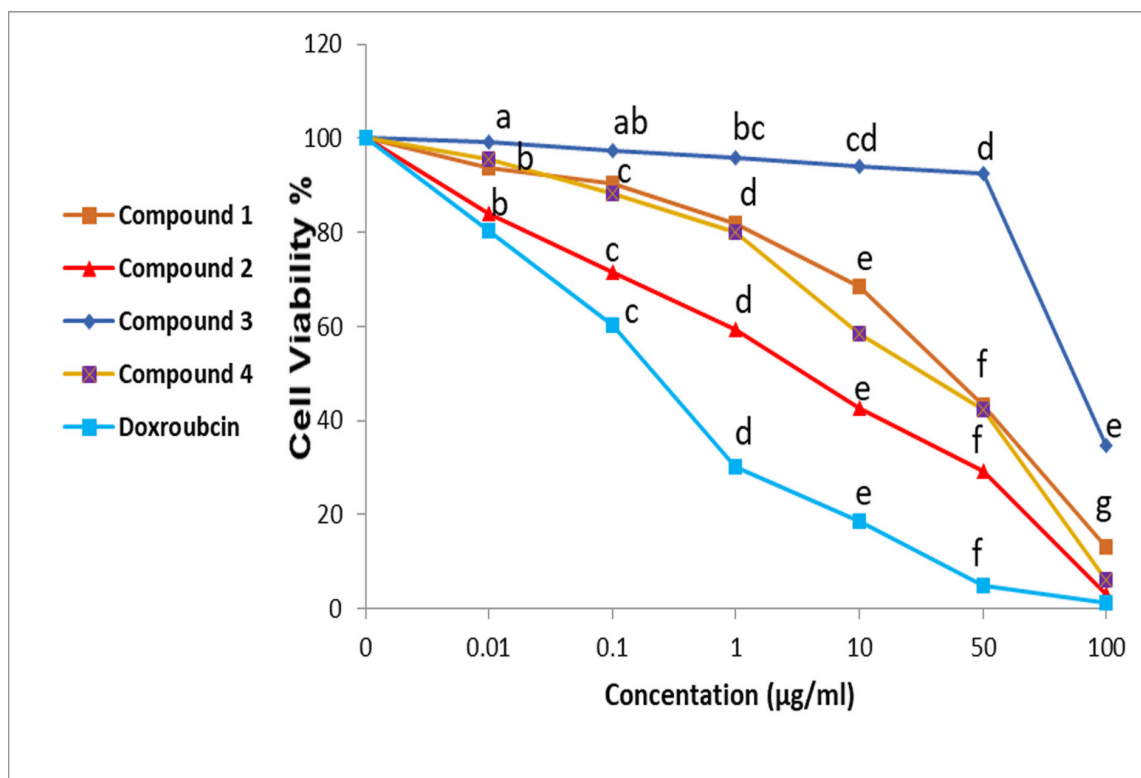


Fig. 8. Dose-response curve of the isolated compounds (1-4) toward MDA-MB-231 cancer cells [Letters (a to g) indicate significant differences  $P < 0.05$  (ANOVA after Tukeys test analysis)]

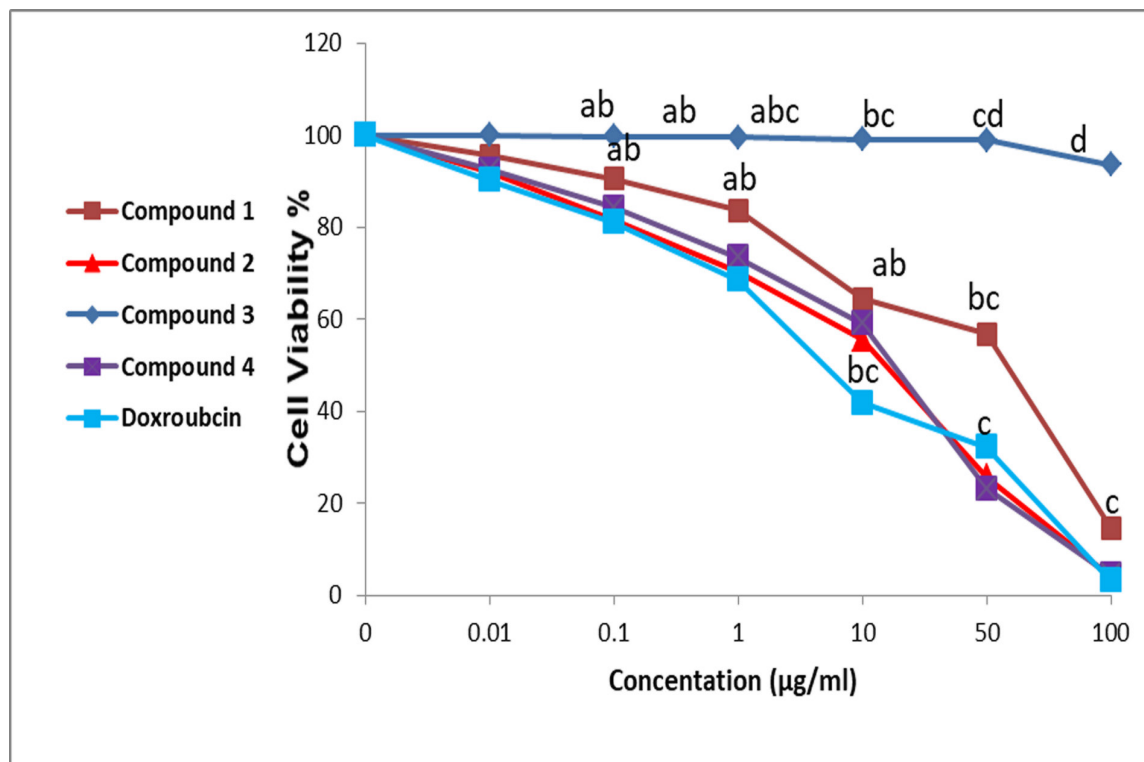


Fig. 9. Dose-response curve of the isolated compounds (1-4) toward SKOV 3 cancer [Letters (a to d) indicate significant differences  $p < 0.05$  (ANOVA after Tukeys test analysis)]

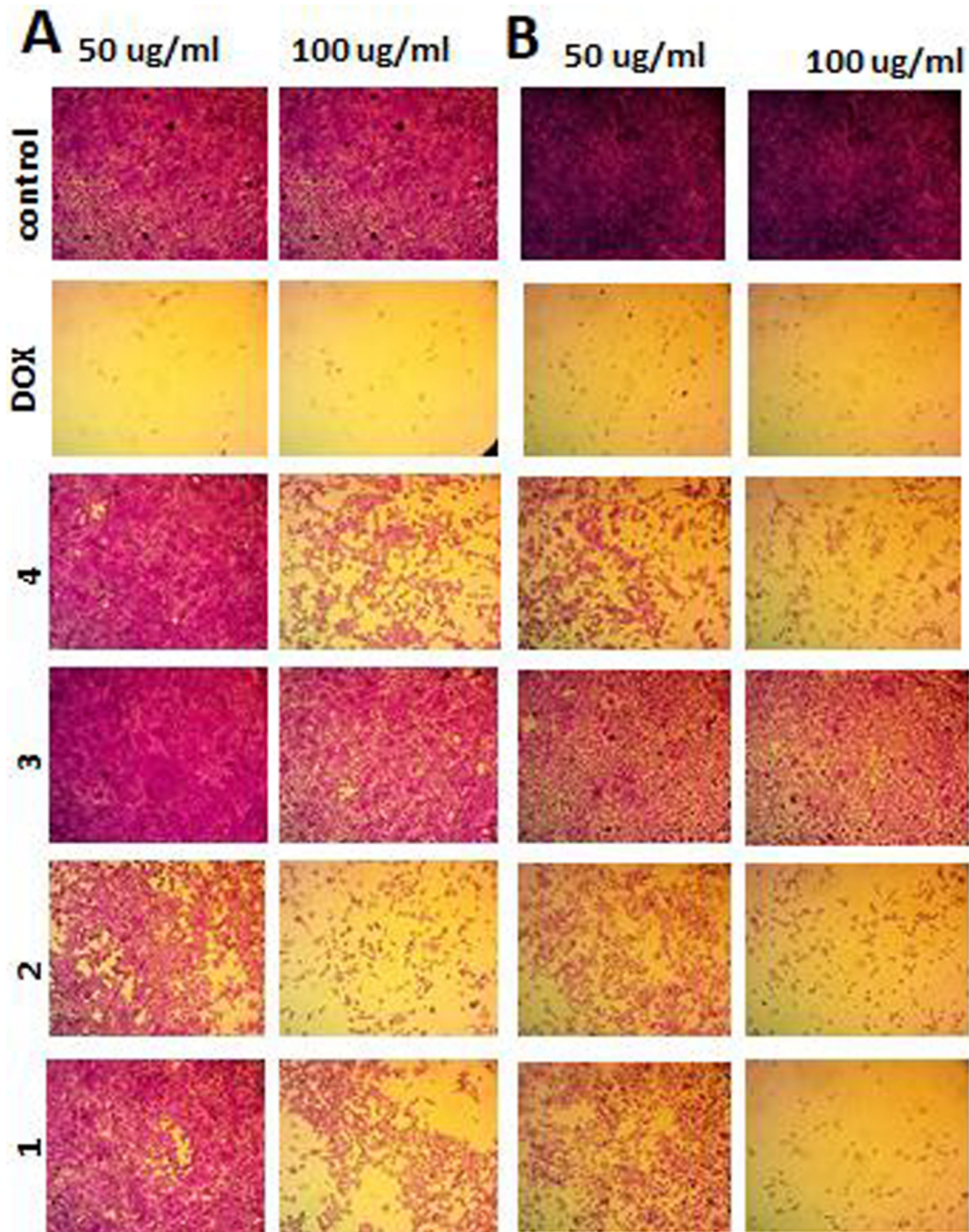


Fig. 10. Effect of compounds (1-4) in 50&100µg/mL against MDA-MB-231 (A) and SKOV 3 (B) cancer cells

#### Cytotoxicity and structure relationship

In the current study, it is possible to correlate the structural properties of isolated compounds to their cytotoxic activity. Compound **1** is a simple phenolic, whereas (**2-4**) are phthalate esters, indicating that the ortho and/or para substitutions of the aromatic ring of (**1-4**) with diverse function groups serve a significant role as a critical pharmacophore for displaying cytotoxic activity, as well as attributing the difference in their

efficacy. Compound **2** ( $IC_{50}$  0.54, 0.75) was the most potent cytotoxic activity, showing a slightly more active than **4** ( $IC_{50}$  0.62, 0.81) toward MDA-MB-231 and SKOV 3 cancer cells respectively (Figs. 8-10). The minor difference in cytotoxicity of **2** and **4** could be explained based on the difference in aromatic function groups of **2** and **4** (Fig. 1). The two butyl moieties of the diester of **2** instead of the pentyl group of monoester with free carboxylic one in **4** slightly enhance the

cytotoxicity of **2** more than **4** against both tested cancer cells (**2** vs **4**) (Figs. 1, 8-10). However, the presence of two isooctyls as high-length alkyl groups of **3** highly reduced its cytotoxicity against both tested cancer cells compared to **2** and **4** (**3** vs **2** & **4**) (Figs. 1, 8-10). On the other hand, the phenolic compound **1** has less cytotoxicity than **2** and **4** against both cancer cells in this study, indicating the butyl diester group of **2** and the pentyl monoester with free carboxylic ones of **4** highly enhanced their cytotoxicity more than the phenolic one (**1** vs. **2** & **4**) (Figs. 1, 8-10). In contrast, phenolic compound **1** is more cytotoxic against both cells than phthalate diester **3**, which has high-length isooctyl groups, reflecting the high-length alkyl diester group highly reduced the cytotoxicity less than the phenolic one (**1** vs. **3**) (Figs. 1, 8-10).

Hence, the cytotoxic activity of **1** as a phenolic derivative was shown to be highly reliant on their phenolic unit. Also, these results showed the influence of variation in the length of the alkyl chain of phthalate esters **2-4** was observed to have a significant effect on each type of ester, whether mono or diesters. These findings may be demonstrated by the drug absorption process, which depends on its lipophilicity and hydrophilicity equilibrium. A drug must be able to interact with both lipophilic (such as membranes) and hydrophilic (such as cytoplasm) environments to reach its site of action. The lipophilicity of the ester is a significant factor, which is predicted to rise with alkyl chain length and results in a greater affinity for the interior (hydrophobic) portion of the lipid bilayer, therefore affecting the compound's location within the cell (Masaki et al., 1997). Recently, it was found that the lipophilicity of ester compounds can be affected by their cytotoxicity, which may be significantly reduced when the ester group is replaced by a hydrophilic moiety (Nam et al., 2001).

#### *Proposed biosynthetic pathway of isolated compounds (1-4)*

Two different biosynthetic routes can be used in plants to produce phenolic compounds: through acetate or shikimic acid from carbohydrates (Geissman & Crout, 1969). The shikimic acid pathway is a common biosynthetic pathway to produce specific secondary metabolites of plants, prokaryotes, and fungi (Richards et al., 2006). However, some authors highlight that bacteria, fungus, and algae rarely produce phenolic

compounds (Natori, 1975). Upon further survey of the literature, we discovered that phenolic secondary metabolites have been isolated in several endophytic microorganism cultures. Sometimes the phenolic metabolites that plants first biosynthesized were also found in their endophyte cultures (Huang et al., 2007). As a result, it is proposed that the biosynthesis of **1** involves the chain extension of 4-hydroxycinnamoyl CoA with malonyl CoA, as well as alkylation, reduction, condensation, oxidation, decarboxylation, and rearrangement reactions (Fig. 11).

On the other hand, phthalates are phthalic acid esters that are naturally occurring bioactive substances in fungi and plants. The benzene dicarboxylic acid and two side chains make up phthalates essential chemical basic structure. These side chains are constituted of chemical groups, including alkyl, phenyl, benzyl, cycloalkyl, and alkoxy (Liang et al., 2008). The accumulation rate of phthalates following an alteration in metabolic activities may be influenced by the fungal and algae growth under stressful conditions, such as light, nutrients, water, temperature, etc. (Staples et al., 1997; Tian et al., 2016). Fungi can synthesize various phthalates by catalyzing the reactions of multiple substrates with their secreted crude enzymes largely through the shikimic acid pathway (Casserly et al., 1983). The protocatechuic acid, a shikimate pathway product, is a significant precursor of phthalic acid, which can be esterified with butyl alcohol, diisooctyl alcohol, and ethyl propyl alcohol to generate compounds **2**, **3**, and **4**, respectively (Fig. 11).

#### *The origin of phthalate acid esters (PAEs)*

Phthalate acid esters (PAEs) are common substances employed for many years as plasticizers in polymers (Thiemann, 2021). These substances have been significantly linked to the appearance of plastics like polyvinyl chloride as highly mobile in the environment and generated by industrial products (Lorz et al., 2007). As a result, quantifiable amounts of PAEs build up in surroundings like the air, soil, water, and sediments, leading to a high level of human exposure. Therefore, PAEs are also clearly identifiable in breast milk (Fan et al., 2019), blood (Albro & Corbett, 1978), and urine (Philips, 2020) of humans. In general, most of these substances are commonly detected as synthetic plasticizers, and widely recognized as having anthropogenic rather than biogenic origins.

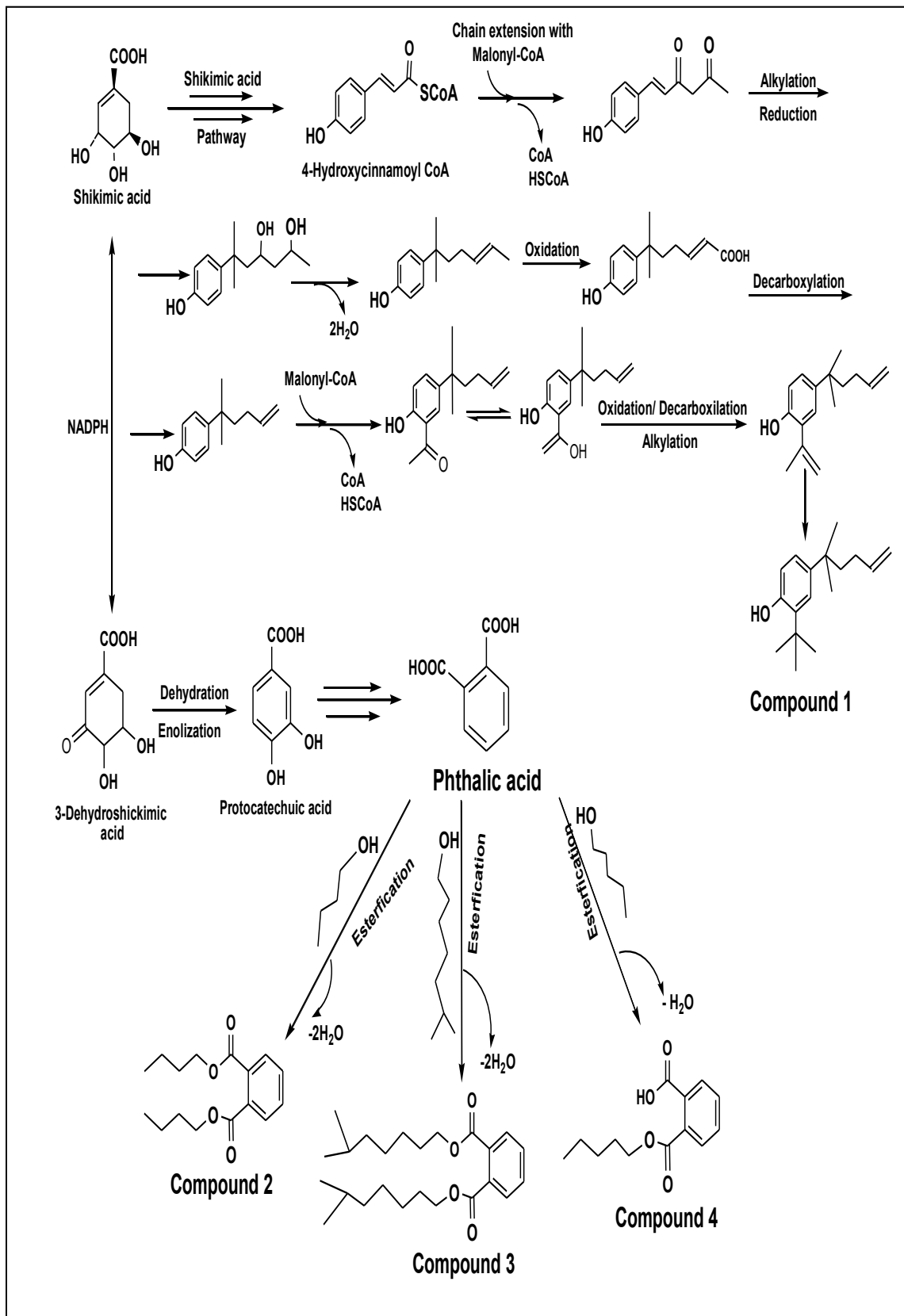


Fig. 11. Proposed scheme of biosynthesis pathway of compounds (1-4)

However, over the past few decades, some bioactive phthalate derivatives have been discovered in unpolluted extracts of natural resources like plants, animals, and microorganisms (Pietra, 1997; Lee, 2000; Zhang et al., 2018). These findings generated a significant discussion regarding the origin of the substance and prompted the debate if it was a true pollutant or a natural metabolite with some biological activities. Therefore, the natural abundance of  $^{14}\text{C}$  content of the relative derivatives of *Ulva* sp., green algae, and two edible brown algae, *Undaria pinnatifida*, and *Laminaria japonica*, were investigated and provided evidence that phthalates can be produced naturally and appropriately (Namikoshi et al., 2006).

Furthermore, it was discovered that naturally occurring filamentous fungal strains (*Trichoderma asperellum* PTN7, *Penicillium lanosum* PTN121, and *Aspergillus niger* PTN42) cultivated in either artificial medium or natural water-produced dibutyl phthalate (DBP) through shikimic acid pathway as microbial secondary metabolites (Tian et al., 2016). As far as we know, since 1979, up to 35 phthalate analogs have been isolated and recognized from various organisms (Zhang et al., 2018, Ortiz & Sansinenea, 2018).

## Conclusion

In this study, we successfully isolated four interested compounds from the thermophilic endophytic fungus *G. thermotolerans*, which was obtained from *E. geniculata*. The identification of these compounds was accomplished through the utilization of 1D and 2D NMR spectroscopy, and mass spectroscopic analyses. The compounds identified are as follows: 2-tert-butyl-4-(2-methylhex-5-en-2-yl) phenol (1), dibutyl phthalate (2), diisooctyl phthalate (3), and 2-(pentyloxy-carbonyl) benzoic acid (4). We conducted in vitro assessments of these compounds, to evaluate their properties, specifically focusing on their antibacterial, antioxidant, and cytotoxic activities. Notably, 2-tert-butyl-4-(2-methylhex-5-en-2-yl) phenol demonstrated inhibition of the growth of *S. aureus* and *S. epidermidis*. Moreover, dibutyl phthalate (2) exhibited significant inhibition of spore germination of *F. solani*. Diisooctyl phthalate displayed the highest DPPH radical scavenging activity, reaching 72.4% at a 500  $\mu\text{g}/\text{ml}$  concentration. On the other hand, both dibutyl phthalate (2) and 2-(pentyloxy-carbonyl) benzoic

acid exhibited potent cytotoxic effects against MDA-MB-231 and SKOV 3 cancer cells.

*Conflict of interest:* The authors confirm that there are no conflicts of interest associated with this publication.

*Authors' contributions:* Noha M. Kamel: Practical work, manuscript preparation, explanation of data, statistical analysis, and publication. Fatma F. Abdel-Motaal: Research point, fungal identification, explanation of data, manuscript preparation and publication. Soad A. El-Zayat: Research point, fungal Identification, explanation of data, manuscript preparation and publication. Abou El-Hamd H. Mohamed: Research Point, explanation of compound structures, manuscript preparation, explanation of data and publication. Shinji Ohta: Explanation and confirmation of compound structures and publication. Taha A. Hussien: Explanation and confirmation of compound structures, statistical analysis, biosynthesis scheme and publication.

*Ethics approval:* Not applicable (this Study does not involve human participants, human data, or human tissue). Compliance with Ethical Standards.

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### مستقلب ثانوي جديد له أنشطة مضادة للميكروبات ومضادات الأكسدة والسامة للخلايا من الفطر الداخلي جيمنواسكس ثيرموتلورانس

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تم عزل مستقلب جديد، وهو مشتق فينولي، 2- ثالثي بيوتيل -4- (2- ميثيل هكسا-5-اين-2- بيل) فينول (1) مع ثلاثة فثالات معروفة (2-4) لأول مرة من الفطر الداخلي جيمنواسكس ثيرموتلورانس الذي تم تجميعه من الجذور والسيقان والأوراق من نبات الايفوريبيا. تم تحديد التركيب عن طريق قياسات الطيف الكتلي والرنين النووي المغناطيسي عالي المجال (1D, 2D).

تم تقييم الخصائص المضادة للبكتيريا، ومضادات الأكسدة، والسامة للخلايا لهذه المستقلبات في المختبر. المركب (1) أعاق بشكل كبير نمو المكورات العنقودية الذهبية والمكورات العنقودية الجلدية مع الحد الأدنى من قيم التركيز المثبط 9.0 و 10.5 ميكرومتر على التوالي.

في المقابل أظهرت كل من ابشريشيا كولاي و كليبسيلا نيمونيا وكانديدا البيكا مقاومة اتجاه المركبات المعزولة من (1-4) التي تظهر نشاطا منخفضا ضد الكانديدا سيفيري. على الرغم من أظهر ثنائي بيوتيل فثالات (2) تثبيطاً قوياً لإنبات جراثيم الفيوزاريم سولاني بنسبة (90.8%) عند أعلى تركيز 2.5 ملغم/مل. علاوة على ذلك، أظهر ثنائي ايزواوكتيل فثالات (3) أعلى نشاطا ل DPPH بنسبة 72.4% عند تركيز 500 ميكروجرام/مل.

من ناحية أخرى، أظهر كل من ثنائي بيوتيل فثالات (2) و 2- (بنتيلوكسي كربونيل) حمض البنزويك (4) سمية خلوية قوية ضد الخلايا السرطانية للثدي والمبيض. بالإضافة إلى ذلك، تمت مناقشة مسارات التخليق الحيوي المقترحة للمركبات المعزولة (1-4) بالإضافة إلى العلاقة بين البنية والنشاط المضادة للميكروبات ومضادات الأكسدة والسامة الخلوية.