# Biologically Active Metabolites from *Penicillium* sp., An Endophyte Isolated from *Glaucium arabicum*

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**Abstract:** A heterodimericxanthone, secalonic acid G **(1)**, a diketopiperazine alkaloid, 3-[(1H-indol-3-yl) methyl]-6-benzylpiperazine-2,5-dione **(2)** and ergosterol **(3)** were isolated from the solid rice culture of a *Penicillium* sp., an endophyte isolated for the first time from both the stems of *Glaucium arabicum* Fres. and the Papaveraceae. The structures were elucidated on the basis of 1D and 2D NMR data. Both compounds **1** and **2** were evaluated for their antioxidant, antibacterial, anti-melanogenesis, cytotoxic, collagen production promoting and hyaluronic acid production promoting activities. Secalonic acid G **(1)** demonstrated a strong and selective antibacterial activity against *S. aureus*, a potent cytotoxic activity against both B16 melanoma and RBL-2H3 cell lines and a significant antioxidant activity. Both compounds showed an inhibitory effect on the production of both hyaluronic acid and collagen but this effect is accompanied by a significant and important increase in the cell viability suggesting their effective use for wound healing.
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## 1. Introduction

Endophytic fungi, the common plant microorganisms, exist in the living tissues of the host plant and do so in a variety of relationships as symbiotic relationship (Strobel & Diasy, 2003). Only a few of these plants have been completely studied relative to their endophytic biology. Secondary metabolites produced by endophytic fungi isolated from plants growing in different ecosystems are considered effective candidates for treating different diseases. Plants with ethnobotanical uses are likely candidates for study, since the medical uses to which the plant may have been selected may be related more to its population of endophytes than to the plant biochemistry itself (Strobel & Daisy, 2003; Yu *et al*., 2010).

Plants belonging to family Papaveraceae are rich in alkaloids that exhibit versatile biological activities as antitussive, antimicrobial, antispasmodic, antihistaminic, anti-inflammatory, cytotoxic, anti-platelet aggregation activities and in the treatment of intestinal disorders (Brossi, 1986). Although plants of Papaveraceae were extensively studied for their alkaloidal content, no previous research dealt with the isolation and identification of endophytic fungal strains living within these plants. Consequently, there is a lack of knowledge about both the endophytes inhabited in the Papaveraceae plants and the chemistry and biology of the secondary metabolites produced by the cultures of these endophytes.

*Glaucium arabicum* Fres. (Papaveraceae) is a rare perennial herb endemic to Sinai Peninsula where it is locally known as No'maan or Ne'man (Täckholm, 1974; Boulos, 1999 & 2009). It is used in the folk medicine of the Bedouins in Sinai for the treatment of eye and skin infections (Khafagy & Dewedar, 2000). Being a desert plant subjected to stress conditions as high temperature and low water supply as well as its local medicinal uses, suggested *G. arabicum* to be a promising source for the isolation of endophytic fungi that could be a source for secondary metabolites with interesting chemical structures and biological activities.

The present study is concerned with the isolation, purification and identification of endophytic fungi of *G. arabicum* stems, followed by the isolation, purification and structural elucidation of the secondary metabolites produced by the solid rice culture of the isolated endophytic fungus. The isolated compounds were also tested for various biological activities.

## 2. Material and Methods

### *2.1. General*

UV spectra ($\lambda_{max}$) was carried out on UV-visible spectrophotometer (Shimadzu 1601 PC, model TCC-240A, Japan) using spectroscopic methanol. IR spectra ($cm^{-1}$) was carried out on Infra-red spectrophotometer, ThermoFisher Scientific, Nicolet is 10 (USA) using KBr pellets. The $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded on Bruker DPX 300 or

AVANCE DMX 600 NMR spectrometers. HRESIMS was measured on a Micromass Qt of 2 mass spectrometer. Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter, measured using a 0.5 mL cuvette with 0.1 dm length. The angle of rotation was measured at the wavelength of 546 and 579 nm of a mercury vapor lamp at room temperature (25º C).

***2.2. Fungal material***

*Glaucium arabicum* Fres. herb was collected from Sinai, Deir El Rabba, near St. Cathrine, Egypt on May 2013. The plant identity was confirmed by St. Cathrine Herbarium staff members. Fresh stem parts were cut into very small pieces, washed with sterilized water, and then the surface was thoroughly treated with 70% ethanol for 1-2 minutes and air dried under the flow hood to avoid surface contaminating microbes. After this process, stem fragments were inoculated in Petri dishes containing Malt agar (MA) medium (Kjer*et al*., 2010), and incubated for five days till the fungal hyphae almost covered the surface of the malt agar plate.

The individual strains were isolated by transferring hyphae tips growing out with a sterile loop onto a fresh malt agar dish. For purification of the fungal strains this step was repeated several times until the colony was supposed uniform.

The isolated strain was identified according to a molecular biological protocol by DNA amplification and sequencing of the ITS region as described by Kjer *et al*., 2010. The GenBank accession number of the fungus is (GenBank: KM108340).The name of the strain is *Penicillium* sp. gl.1.

**2.3.** ***Production and isolation of fungal metabolites:***

The isolated fungus was grown on solid rice prepared by autoclaving 100 g of rice and 100 mL of water in a 1 L flask. The fermentation was performed in five flasks and left for 30 days at room temperature. To each 1L flask 400 ml EtOAc were added and left for 3 days to allow complete extraction. After filtration, re-extraction was done with fresh EtOAc three times till exhaustion. The combined EtOAc extracts were washed with distilled water and then evaporated under reduced pressure till dryness. The dry residues were dissolved in 90% MeOH and defatted by shaking with n-hexane to yield 750 mg defatted MeOH extract. The latter was fractionated on Diaion HP 20 by elution with distilled $H_2O$/MeOH (100:0, 90:10, 70:30, 50:50, 25:75 and 0:100), followed by methanol : acetone (50:50) and finally with 100% acetone.

Fraction eluted with $H_2O$ : MeOH (25:75) was purified on sephadex LH 20 using methylene chloride : methanol (98:2 & 93:7). Fractions eluted with methylene chloride : methanol 93:7 were collected together, evaporated till dryness yielding yellow needles of compound **1** (25 mg).

Fraction eluted with $H_2O$ : MeOH (0:100) was purified on silica gel 60 column eluted with pet.ether : ethyl acetate (100:0 to 10:90). Fractions eluted with pet. ether : ethyl acetate 35:65 were collected together, evaporated till dryness to yield a white powder of compound **2** (7 mg). By the same way fractions eluted with pet. ether : ethyl acetate 25:75 were collected, evaporated, dried to yield compound **3** (2 mg) as a white powder.

***2.4. Identification of the isolated compounds***

***Compound 1***

Compound **1** (25 mg) was isolated as yellow needles. $R_f$ = 0.20 ($CH_2Cl_2$ : $CH_3OH$, 9:1), $[\alpha]_D$ – 93 (Conc. 0.7 gm/100 ml chloroform at 20º). Its molecular formula was determined to be $C_{32}H_{30}O_{14}$ from the $[M+H]^+$ peak at m/z 639.1710 (calculated, 639.1714) in the HRESIMS. The IR spectrum showed absorption bands at 3524, 2927, 1747, 1592, 1442, 1232, 1039 $cm^{-1}$. The UV spectrum showed absorption maxima at 340.4, 295.0, 279.4, 270.4 nm. $^1$H NMR (600 MHz, $CDCl_3$): 1.17 (3H, d, *J*=2.64, H-11′), 1.18 (3H, d, *J*=2.88, H-11), 2.12 (1H, m, H-6), 2.32 (1H, dd, *J*=19.2, 10.6, H-7′), 2.40 (1H, dd, *J*=18.9, 6.2, H-7), 2.42 (1H, m, H-6′), 2.53 (1H, dd, *J*=19.0, 11.2, H-7), 2.54 (1H, s, OH-5), 2.74 (1H, dd, *J*=19.2, 6.3, H-7′), 2.79 (1H, s, OH-5′), 3.72 (1H, s, H-13′), 3.73 (1H, s, H-13), 3.93 (1H, dd, *J*=11.3, 2.1, H-5′), 4.12 (1H, s, H-5), 6.58 (1H, d, *J*=8.40, H-4), 6.63 (1H, d, *J*=8.46, H-4′), 7.43 (1H, d, *J*=8.46, H-3), 7.46 (1H, d, *J*=8.46, H-3′), 11.74 (1H, s, OH-1′), 11.87 (1H, s, OH-1), 13.79 (1H, s, OH-8), 13.97 (1H, s, OH-8′). $^{13}$C NMR (150 MHz, $CDCl_3$): 17.7 (C-11′), 18.1 (C-11), 28.6 (C-6), 29.4 (C-6′), 32.7 (C-7), 36.4 (C-7′), 53.4 (C-13′), 53.7 (C-13), 71.5 (C-5), 77.3 (C-5′), 84.88 (C-10′a), 84.9 (C-10a), 100.1 (C-8′a), 101.7 (C-8a), 107.0 (C-9′a), 107.1 (C-9a), 107.68 (C-4), 107.7 (C-4′), 118.3 (C-2), 118.9 (C-2′), 139.9 (C-3), 140.3 (C-3′), 157.3 (C-4a), 158.4 (C-4′a), 159.5 (C-1′), 159.6 (C-1), 170.4 (C-12), 171.4 (C-12′), 177.7 (C-8), 179.9 (C-8`), 187.3 (C-9′), 187.8 (C-9).

***Compound 2***

Compound **2** (7 mg) was isolated as a white powder. $R_f$ = 0.25 (Pet. ether : EtOAc, 4:6) $[\alpha]_D$ – 179 (conc. 0.3 gm/100 ml methanol at 20º). Its molecular formula was determined to be $C_{20}H_{19}N_3O_2$ from ESI-MS data (334.0 $[M+H]^+$ and 332.4 $[M-H]^-$. The IR spectrum showed bands at 1457, 1670, 3421$cm^{-1}$. The UV spectrum showed absorption maxima at 295.2, 284.6, 269.2 nm. $^1$H NMR (300 MHz, $CDCl_3$): 1.85 (1H, dd, *J*=13.38, 7.05, H-7″), 2.50 (1H, m, H-7″), 2.52 (1H, m, H-8″), 2.79 (1H, dd, *J*=16.47, 4.41, H-8″), 3.85 (1H, m, H-6), 3.97 (1H, m, H-3), 6.71 (2H, m, H-3″, 5″), 6.96 (1H, d, *J*=2.04, H-2`), 6.98 (1H, ddd, *J*=7.44, 7.35, 1.2, H-5′), 7.07 (1H, ddd, *J*=7.56,

7.41, 1.7, H-6′), 7.16 (1H, m, H-4″), 7.18 (2H, m, H-2″, 6″), 7.32 (1H, dd, *J*=8.5, 1.0, H-7′), 7.48 (1H, bd, *J*=7.8, H-4′), 7.71 (1H, d, *J*=2.76, H-1), 7.90 (1H, d, *J*=2.55, H-4), 10.88 (1H, s, H-1′). $^{13}$C NMR (75 MHz,$CDCl_3$): 29.7 (C-8′), 38.8 (C-7″), 55.2 (C-3), 55.6 (C-6), 108.8 (C-3′), 111.3 (C-7′), 118.4 (C-5′), 118.7 (C-4′), 120.9 (C-6′), 124.4 (C-2′), 126.4 (C-4″), 127.5 (C-3′a), 128.0 (C-2″, 6″), 129.7 (C-3″, 5″), 136.0 (C-7′a), 136.5 (C-1″), 166.2 (C-5), 166.8 (C-2).

***2.5. Reagents and cell lines used in biology :***

***2.5.1 Chemical reagents for biological assays***

NaOH and DMSO were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma (St. Louis, MO), EMEM from Nissui Chemical Co (Osaka, Japan). Other chemicals were of the highest grade commercially available.

***2.5.2 Cell lines***

Mouse melanoma cell line, B16, and rat basophilic cells, RBL-2H3, were obtained from RIKEN Cell Bank. The cells were maintained in EMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL pencillin and 100 U/mL streptomycin. The cells were incubated at 37$^o$C in a humidified atmosphere of 5% $CO_2$.

***2.6. Biological assays***

***2.6.1 Melanin biosynthesis assay***

This assay was carried out as described by (Ashour *et al*., 2013). The cells were placed in two plates of 24-well plastic culture plates (one plate for determining melanin content and the other for cell viability) at a density of 1 x $10^5$ cells/well and incubated for 24 h in media prior to being treated with the samples. After 24 h, the media were replaced with 998 µL of fresh media and 2 µL of the test sample at the final concentration of 5, 10, 20 µg/ mL (n = 3). At the same time, negative control (2 µL DMSO) and positive control; Arbutin at concentration 50 mg/mL in DMSO were tested. The cells were incubated for an additional 48 h, and then the medium was replaced with fresh medium containing each sample. After 24 h, the remaining adherent cells were assayed. To determine the melanin content (for one plate) after removing the medium and washing the cells with PBS, the cell pellet was dissolved in 1.0 mL of 1 N NaOH, kept overnight in dark then measured by using a microplate reader at 405 nm to determine the melanin content. The results from the cells treated with the test samples were analyzed as a percentage of the results from the control culture. The cell viability was determined using MTT assay. So, for the other plate, 50 µL of MTT reagent in PBS (5 mg/mL) were added to each well. The plate was incubated in a humidified atmosphere of 5% of $CO_2$ at 37$^o$C for 4 h. After the medium was removed, 1.0 mL isopropyl alcohol (containing 0.04 N HCl) was added, and the absorbance was measured at 570 nm after overnight keeping in dark.

***2.6.2 Cytotoxicity against RBL-2H3 cell lines:***

Cytotoxicity of the tested compounds against allergic cells (RBL-2H3 cells) was done using MTT technique as described in the melanin biosynthesis assay.

***2.6.3 Antibacterial assay***

The antibacterial assay was carried out based on the methods described by (Tanaka *et al*., 2013). A single colony of the test strain was taken and 5 mL of nutrient broth (NB) medium was added to it. This culture was incubated at 37°C ± 1°C, 1150 rpm for 18 hours. Then, the turbidity of the bacterial suspension was measured by a spectrophotometer at 630 nm and adjusted to 0.4 by adding NB medium. The concentration of the bacterial suspension was $10^8$ CFU/mL so; sterilized water was added to culture suspension to prepare bacterial concentration of $10^5$ CFU/mL which was used for the following antibacterial assay. Each sample was dissolved in DMSO at maximum concentration (1600 µg/mL). In each well of a 96-well plate, 133.5 µL of NB medium, 15 µL of bacterial suspension, and 1.5 µL of DMSO were added with or without each sample. Also, sorbic acid (400 µg/ml) was used as a positive control.

The plate was incubated at 37°C ± 1°C, 1160 rpm for 18 h. Finally, bacterial growth was measured by a micro-plate reader (630 nm). The statistical difference between the control and each sample was determined by Student's t-test. The MIC (minimum inhibitory concentration) was the lowest concentration of the test extract that completely prevented growth until 18 h. MBC (minimum bactericidal concentration), was determined as follows; after determining the MIC, 20 µL aliquot was taken from each well and added into 180 µL of fresh medium. Then, 100 µL of the suspension was used to do a subculture on nutrient agar (NA) plate. After 24 h incubation at 37°C ± 1°C, the growth colony was visibly evaluated. No growth of colony on the agar plate was regarded at the minimum bactericidal concentration.

***2.6.4 Hyaluronic acid (HA) ELISA assay on (NHDF-Ad):***

This kit is an enzyme-linked binding protein assay that uses a capture molecule known as hyaluronic acid binding protein (HABP). After growing of the fibroblast cells, they are incubated in HABP-coated micro well plate. Properly samples and HA reference solution are added to this plate, allowing HA present to react with the immobilized binding protein. After removal of unbound molecules by washing, HABP conjugated with horseradish peroxidase (HRP) solution is added to the microwells

to form complexes with bound HA. Following another washing step, a chromogenic substrate of tetramethyl benzidine and hydrogen peroxide is added to develop a colored reaction. The intensity of the color is measured in optical density units with a spectrophotometer at 450 nm. The higher color intensity, the higher ability of the sample to produce hyaluronic acid.

Firstly, sample concentration that showed no cell toxicity to NHDF-Ad (normal-human dermal fibroblast from adult skin) after 48 hours incubation with sample was determined by MTT assay. Hyaluronic acid production-promoting effect on NHDF-Ad was evaluated at concentration of the samples that showed no cell toxicity. The amount of hyaluronic acid produced by NHDF-Ad was measured using an ELISA kit. Previously, NHDF-Ad cells ($2.0\times10^4$ cells/well) were cultured in a 96-well plate with 100 µL of DMEM supplemented with 10% FBS. After 24 h incubation, test samples [0.5 µL/well, DMSO as a control, were added to the cells soon after the medium was replaced with 100 µL of DMEM supplemented with 0.5% FBS. After 48 h incubation, the hyaluronic acid amount produced by NHDF-Ad in the supernatant was quantified by using a human hyaluronic acid ELISA kit (ACEL, Japan). A standard curve was made in the same ELISA plate (n=3). On the other hand, the cell viability of the remaining cells was measured by MTT assay (n=3). The statistical difference between the control and each sample was determined by Student's t-test.

***2.6.5 Collagen ELISA assay on (NHDF-Ad):***

The assay was performed to screen for promoters of collagen production of dermal fibroblasts. NHDF-Ad was seeded on 96-well plates ($2.0\times10^4$ cells/well) 24h before the treatment. The medium was replaced with a mixture of 0.5 µL of DMSO solution of the compounds and 100 µL of DMEM supplemented with 0.5% FBS. After 72 h cultivation, the amount of collagen in the medium was measured using a human collagen type I ELISA kit (ACEL, Japan). The cells remaining in the 96-well plate were subjected to MTT assay. The solvent used for dissolving the sample was used as a negative control, and ascorbic acid (17.6µ g/mL, 100 µM) was used as a positive control.

Firstly, sample concentration that showed no cell toxicity to NHDF-Ad (normal-human dermal fibroblast from adult skin) after 48 hours incubation with sample was determined by MTT assay (the same experiment for HA assay).

***2.6.6 Antioxidant activity screening assay using ABTS***

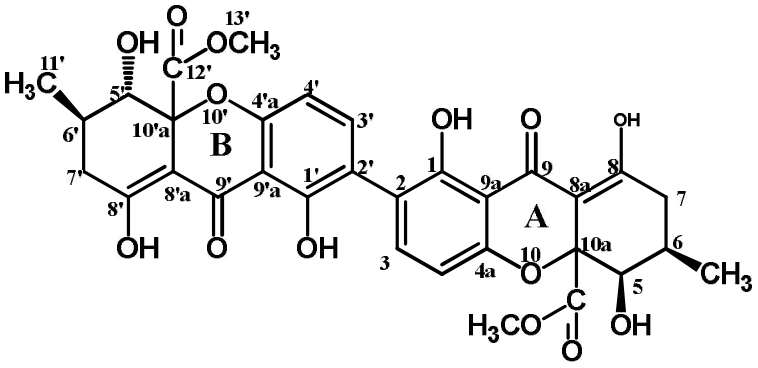
The antioxidant activity was evaluated using a colorimetric method described by Lissi *et al*., 1999.

## 3. Results and Discussion

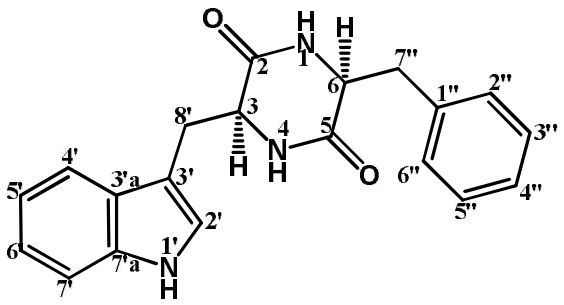
An endophytic fungus was isolated from the fresh stems of *G. arabicum* and identified as a *Penicillum* sp. according to a molecular biological protocol (Kjer*et al*., 2010). Investigation of the ethyl acetate extract of the solid rice culture of *Penicillum* sp. led to the isolation of a heterodimeric xanthone, secalonic acid G **(1)** (El-Elimat *et al*., 2015), a diketopiperazine alkaloid, 3-[(1H-indol-3-yl) methyl]-6-benzylpiperazine-2,5-dione **(2)** (Kimura *et al*., 1995) and ergosterol **(3)** (Tao *et al*., 2013). To the best of our knowledge, the heterodimeric xanthone, secalonic acid G, is reported in the present study for the first time from genus *Penicillum*. Compounds **1** and **2** were tested for their antibacterial, cytotoxic, anti-melanogenesis, collagen production promoting, hyaluronic acid production promoting and antioxidant activities. Compound **1** demonstrated a strong and selective antibacterial activity against *S. aureus*, a potent cytotoxic activity on B16 melanoma cells rather than melanin formation inhibition, a potent cytotoxic effect on RBL-2H3cell line and a significant antioxidant activity compared to ascorbic acid. Compound **2** showed a moderate effect on melanin inhibition with nearly no cytotoxic activity on B16 melanoma cells and a moderate antioxidant activity.

**Compound 1** was isolated as yellow needles. Its molecular formula was determined to be $C_{32}H_{30}O_{14}$ from the $[M+H]^+$ peak at m/z 639.1710 in the HRESIMS. The UV spectrum showed an absorption maximum at 340 nm. The IR spectrum showed bands at 3524 $cm^{-1}$ (OH group), 1747 $cm^{-1}$ (carbonyl moiety), 1613 $cm^{-1}$ (an enolized β-diketone) and 1592 $cm^{-1}$ (hydrogen-bonded carbonyl). The data obtained from The UV and IR spectra suggested that compound **1** may be structurally related to the secalonic acids (Wagenaar & Clardy, 2001; Kurobane & Vining, 1978). The $^{13}C$ NMR spectrum showed 32 carbon signals in pairs with closely related chemical shift values for each pair suggesting that compound **1** is a dimer. Examination of 1D ($^1H$ and $^{13}C$) and 2D ($^1H$-$^1H$ COSY, HMQC and HMBC) NMR spectra of compound **1** revealed the presence of a tetrahydroxanthone dimer consisting of two heteromonomers. Each monomer showed two carbonyls (an acetate and a ketone), a tetrasubstituted aromatic ring, an enol, one methyl group, one methyl ester, one methylene group, two methines and one quaternary carbon. $^1H$ NMR spectrum showed two H-bonded hydroxyl groups ($\delta_H$ 11.87 and 13.79) and one free hydroxyl ($\delta_H$ 2.54) in monomer A and two H-bonded hydroxyl groups ($\delta_H$ 11.74 and 13.97) and one free hydroxyl ($\delta_H$ 2.79) in monomer B. The 2 monomers are linked through C-2, C-2′ linkage as confirmed by the HMBC correlations of H-3 and H-3′ ($\delta_H$ 7.43 and 7.46) with C-2 and C-2′ ($\delta_c$ 118.3 and 118.9), respectively. Some differences were observed

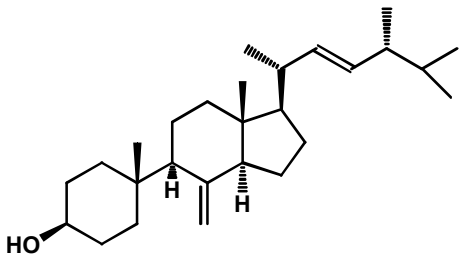
in the $^{1}H$ and $^{13}C$ NMR resonances at C-5 and C-7 positions of both monomers, where the signals of C-5 in monomer A ($\delta_{H/C}$ 4.12, s/ 71.5) were replaced by signals of C-5′ ($\delta_{H/C}$ 3.93, dd, *J*= 11.3, 2.1 Hz /77.3) in monomer B. There is also an upfield shift (3.7 ppm) of C-7 in monomer A ($\delta_c$ 32.7 ppm), compared to that of C-7′ in monomer B ($\delta_c$ 36.4 ppm). The large coupling constant between H-5′ and H-6′, *J*=11.3 Hz indicated a pseudodiaxial orientation of both H-6′ and the α-oriented H-5′ therefore the hydroxyl group (on C-5′) and the methyl group (on C-6′) should be pseudo equatorial (Isaka *et al*., 2005). The upfield shift of C-7 ($\delta_c$ 32.7) in comparison with that of C-7′ ($\delta_c$ 36.4 ppm) is attributed to the γ-*gauche* effect of OH at C-5 (Zhang *et al*., 2008; Barfield & Yamamura, 1990; Li & Chesnut, 1985). The above data together with the negative sign of the optical rotation of compound **1**, $[\alpha]_D$ – 93 (Conc. 0.7 gm/100 ml chloroform at 20º ) indicated that compound **1** is secalonic acid G (5*R*, 6*R*, 10a*S*, 5′*S*, 6′*R*, 10′a*S*) (El-Elimat *et al*., 2015).




Secalonic acid G **(1)**



3-[(1H-indol-3-yl) methyl]-6-benzylpiperazine-2,5-dione **(2)**




Ergosterol **(3)**

**Figure 1.** Structures of compounds **1-3**

**Compound 2** was isolated as a white powder, Its molecular formula was determined to be $C_{20}H_{19}N_3O_2$ from the ESI-MS data (334.0 $[M+H]^+$ and 332.4 $[M-H]^-$). The IR spectrum showed absorption bands at 1457, 1670, 3421 $cm^{-1}$. The UV spectrum showed absorption bands at 295, 284, 269 nm. Examination of 1D ($^{1}H$ and $^{13}C$) and 2D ($^{1}H$-$^{1}H$ COSY, HMQC and HMBC) NMR spectra of compound **2** revealed the presence of a mono-substituted phenyl ring, a piperazine dione ring, an indole moiety and 2 methylenes. The mono-substituted phenyl ring was represented by signals at $\delta_H$ 6.71 (2H, m) (H-3″, 5″), 7.16 (1H, m) (H-4″) and 7.18 (2H, m) (H-2″,6″) correlated to $\delta_c$ 129.7 (C- 3″,5″), 126.4 (C-4″) and 128.0 (C-2″,6″), respectively. The piperazine dione ring is represented by the two secondary amide groups suggested by an infrared band at 1670 $cm^{-1}$ together with signals at $\delta_H$ 7.71(1H, d) (H-1) and 7.90 (1H, d) (H-4) in the $^{1}H$ NMR spectrum and $\delta_c$ 166.2 (C-5) and 166.8 (C-2) in the $^{13}C$ NMR spectrum. The HMBC correlations (Figure 2) observed from $\delta_H$ 7.71 (H-1) to $\delta_c$ 166.8 (C-2) and 55.6 (C-6) and from $\delta_H$ 7.90 (H-4) to $\delta_c$ 166.2 (C-5) and 55.2 (C-3) confirmed the piperazine dione moiety. The indole moiety was represented by the proton at $\delta_H$ 10.88 (1H, s, H-1′), the four aromatic protons at $\delta_H$ 7.48 (1H, *bd*, *J*=7.8, H-4′), 6.98 (1H, *ddd*, *J*=7.44, 7.35, 1.2, H-5′), 7.07 (1H, *ddd*, *J*=7.56, 7.41, 1.7, H-6′) and 7.32 (1H, *dd*, *J*=8.5, 1.0,

H-7′). The HMBC correlations from $\delta_H$ 10.88 (H-1′) to $\delta_c$ 108.8 (C-3′) and $\delta_c$127.5 (C-3′a) and its COSY correlation with $\delta_H$ 6.96 (H-2′) characterized the indole moiety.

The piperazine dione ring is linked to the mono substituted phenyl moiety through a methylene bridge ($\delta_H$ 1.85 and 2.50, $CH_2$-7″), this was deduced from the HMBC correlations from $\delta_H$ 1.85 to $\delta_c$ 166.2 (C-5) of the piperazine dione ring and from $\delta_H$ 2.50 to $\delta_c$ 128.0 (C-2″, 6″) of the phenyl moiety. The indole ring is connected to the piperazine dione ring through a methylene bridge ($\delta_H$ 2.79 and 2.52, $CH_2$-8′) indicated through HMBC correlations from $\delta_H$ 2.52 to $\delta_c$ 166.8 (C-2) of the piperazine dione ring and from $\delta_H$ 2.79 to $\delta_c$ 127.5 (C-3′a) of the indole moiety.

From the above data, compound **2** was identified as the diketopiperazine alkaloid, 3-[(1H-indol-3-yl) methyl]-6-benzylpiperazine-2,5-dione. It was previously isolated from a *Penicillium* sp. (Kimura *et al*., 1995) and known as Cyclo-(L-tryptophyl-L-phenylalanyl).

**Compound 3** was identified as ergosterol depending on the $^1$H NMR spectrum compared to published data (Tao *et al*., 2013).

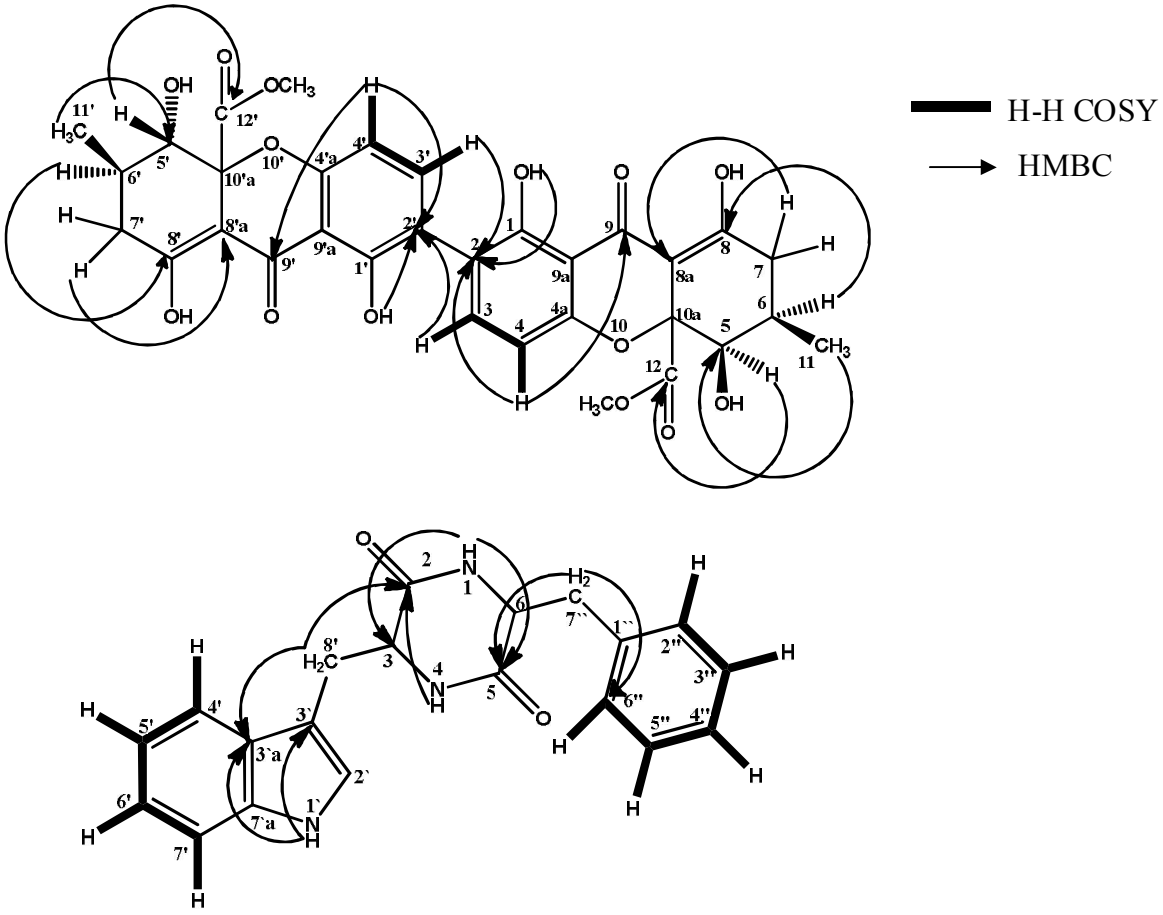



**Figure 2.** Selected $^1$H-$^1$H COSY and HMBC correlations for compounds **1** and **2**

***Biological activities***

***Melanin biosynthesis assay:***

The isolated compounds were assayed by using B16 melanoma cells in order to evaluate the inhibition of melanin formation and cell viability at their maximum solubility (20 µg/mL). The results are shown in figure **3**. The isolated compounds were tested for their ability to inhibit melanin biosynthesis using B16 melanoma cells taking in consideration the cell viability at concentrations of 5, 10 and 20 µg/mL. Compound **1** at all tested concentrations showed cytotoxicity on B16 melenoma cells rather than melanin formation inhibition. Compound **2** at concentrations 20 µg/mL had moderate effect on melanin inhibition (12%) with nearly no cytotoxicity; however other concentrations showed no melanin inhibition activity.

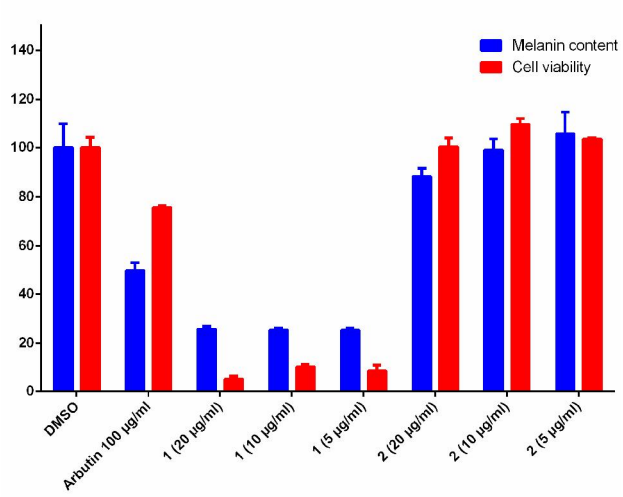



**Figure 3.** Effect of compounds **1** and **2** on melanin biosynthesis and cell viability of B16 melanoma cells.

***Cytotoxicity against RBL-2H3 cell lines:***

The results of this assay are shown in figure **4**. It showed that compound **1** at both tested concentrations (100 and 50 µg/mL) showed a potent cytotoxic activity against RBL-2H3 cells compared to the positive standard gallic acid, however, compound **2** showed no cytotoxic activity. It is worth to mention that compound **1** showed a potent cytotoxic activity against both tested cancer cell lines (B16 melanoma cells and RBL-2H3cells) suggesting its potential anticancer effect.

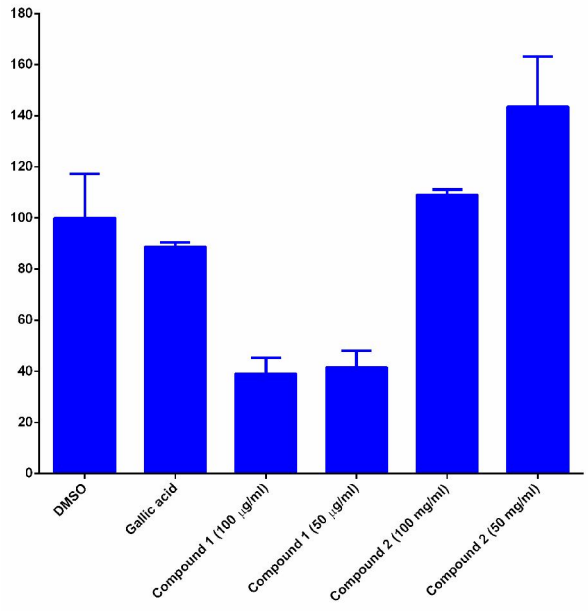



**Figure 4**. Cytotoxic activity of compounds **1** and **2** on RBL-2H3 cell lines

***Antibacterial assay***

Antibacterial activity against *S. aureus* is an important attribute of skin cosmetics, because the proliferation of bacteria causes skin problems such as acne, comedo, papules, cellulitis and allergies (Meng*et al*., 2011; Breuer *et al*., 2002). Therefore the antibacterial activity of the isolated compounds were evaluated and the obtained results are showed in figure **5**. Compound **1** showed the strongest antimicrobial effect against *S. aureus* and did not show any activity against *E. coli* in comparison with the positive control (sorbic acid). Compound **2** didn't show any antibacterial activity against both types of bacteria.

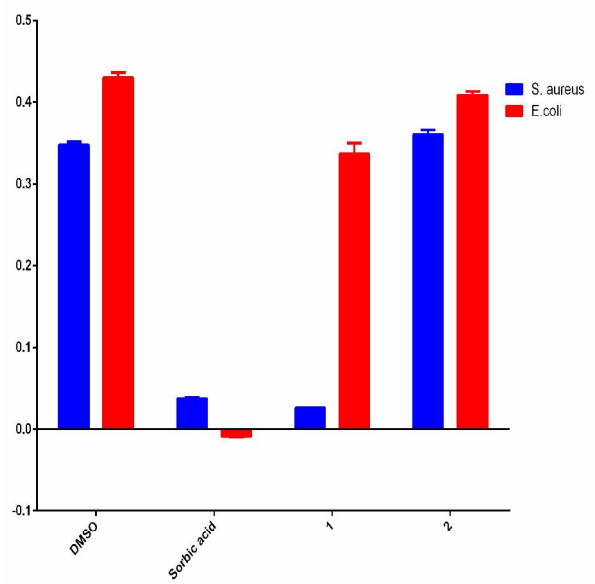



**Figure 5.** Antibacterial activity against *E. coli* and *S. aureus*. The values are represented as the mean ± standard deviation (SD), n=3. Final concentration; 400 µg/mL for sorbic acid and 100 µg/mL for tested compounds. Significant difference between 1% DMSO and each compound was determined by Student's t-test: **P* < 0.05, ***P*< 0.01.

***Hyaluronic acid (HA) ELISA assay on (NHDF-Ad):***

Compounds **1** and **2** were tested for their effect on the production of HA (hyaluronic acid) by the use of HA ELISA assay using Biotech Trading Partners (Encinitas, California) according to manufacturer's instructions. It was found that both compounds **1** and **2** at the tested concentration have an inhibitory effect on the production of hyaluronic acid. From these results, it can be concluded that these compounds can't be used for the production of hyaluronic acid. Although the results are not good from the expected point of view but the good point is that these

compounds raised the cell viability consequently, can be used in the improvement of wound healing. Results are shown in figure **6**.

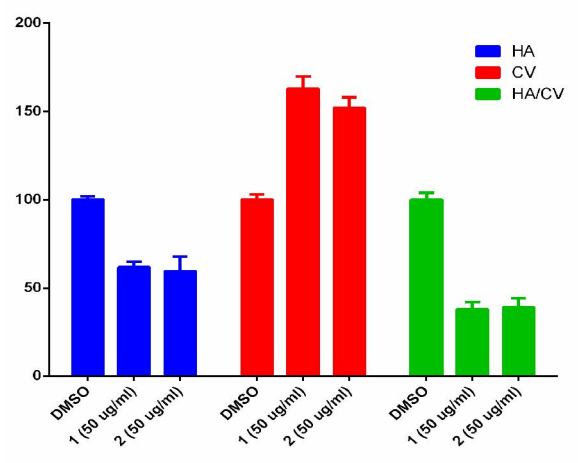



**Figure 6.** Effect of **1** and **2** on the production of hyaluronic acid. The values are represented as the mean ± S.D, n=3

***Collagen ELISA assay on (NHDF-Ad):***

Figure **7** showed the effect of compounds **1** and **2** on the collagen production of NHDF-Ad (CP) and cell viability (CV). To express CP and CV, each control value was set at 100%. Ascorbic acid, a positive control, increased CP (469%) and had no effect on CV (105%). Both tested compounds inhibited collagen production but a significant increase in cell viability (162% and 132%, respectively) was observed. These observations confirmed our previous results that both compounds **1** and **2** can be effective in wound healing.

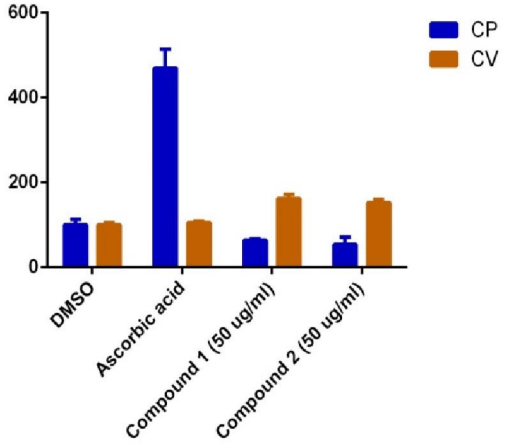



**Figure 7.** Effect of compounds **1** and **2** on collagen production and cell viability. The values are represented as the mean ± standard deviation (SD).

***Antioxidant Activity screening assay by ABTS:***

From the results listed in Table **1**, we can conclude that compound **1** has a strong antioxidant activity compared to that of ascorbic acid, while compound **2** showed moderate antioxidant activity.

Table **1.** Antioxidant activity of compounds **1** and **2** using ABTS assay

| Compounds | Absorbance of samples | % inhibition |
|---|---|---|
| **Control** | 0.514 | 0 % |
| **Ascorbic-acid** | 0.056 | 89.1 % |
| **1** | 0.105 | 79.6 % |
| **2** | 0.244 | 52.5 % |

**4. Conclusion**

Phytochemical investigation of the ethyl acetate extract of the solid rice culture of a *Penicillum* sp. isolated from the stems of *Glaucium arabicum* led to the isolation of secalonic acid G, 3-[(1H-indol-3-yl) methyl]-6-benzylpiperazine-2,5-dione and ergosterol. Secalonic acid G exhibited a strong and selective antibacterial activity against *S. aureus*, potent cytotoxic activity on both B16 melanoma and RBL-2H3cell lines, and a significant and strong antioxidant activity. 3-[(1H-indol-3-yl) methyl]-6-benzylpiperazine-2,5-dione showed a moderate effect on melanin inhibition with nearly no cytotoxicity and a moderate antioxidant activity. Both compounds showed an inhibitory effect on the production of both hyaluronic acid and collagen but this effect is accompanied by a significant and important increase in the cell viability suggesting their effective use for wound healing.

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