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Breast Cancer Migration and Proliferation Inhibitory and Antibiotic Secondary Metabolites from the Egyptian Olive Tree Endophytic Fungus *Penicillium citrinum*

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ABSTRACT

Objectives: The main aim of this study is to discover fungal endophytes associated with the Egyptian native olive tree *Olea europaea* as new sources for breast cancer control and investigate the chemistry and bioactivity of the fermentation metabolites of the identified active fungal species. **Methods**: Screening of multiple cultured Egyptian olive tree-associated fungal endophyte extracts identified *Penicillium citrinum* extract in breast cancer proliferation assays. Bioassay-guided isolation of the fungus *P. citrinum* extract identified four known metabolites **1**-**4**. Their structure identity was established by different 1D and 2D NMR analyses. Compounds were tested for antiproliferative activity against the human breast cancer cell lines BT-474, MDA-MB-231 and MDA-MB-468using MTT assay and antimigratory activity against the human breast cancer cell lines MDA-MB-231 using wound healing assay. The antimicrobial activity of **1**-**3** was assessed using modified Kirby-Bauer disc diffusion method against multiple Gram positive and negative bacteria and fungi. **Results**: NMR analyses identified **1** as the known mycotoxin citrinin and the three different pyrrolidine alkaloids: 2-(hept-5-enyl)-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4*H*-pyrrolo[2,1-*b*]-1,3-oxazine (**2**), scalusamide A (**3**) and perinadine A (**4**). Compounds **2-4** showed significant antiproliferative activity against the human triple negative breast cancer cell line MDA-MB-231 with IC_{50} values of 10.6, 14.8 and 17.7 µM, respectively. Prerinadine (**3**) significantly suppressed the HGF-induced cell migration in a dose dependent manner with IC_{50} 9.7. Citrinin (**1**) exerted remarkable antibiotic activity against Gram (+) and Gram (-) bacteria and was even up to several-fold those of tetracycline standard. **Conclusion:** Egyptian olive tree endophytes are valuable sources for biologically active natural products.

Keywords: *Anti-migratory, Anti-proliferative, Breast cancer, Endophytes, Olive tree, Penicillium citrinum*

INTRODUCTION

Extensive research efforts have considered plants as "bio-factories" of potentially valuable biologically active compounds, with several drawbacks including slow growth rate and ecological damage to several rare plant species. Therefore it is imperative to discover and focus on alternative approaches and resources. Finding new potential sustainable sources of novel bioactive molecules directed researchers to symbiotic microorganisms associated with a wide-range of plant species and are termed as endophytes.[1] Endophytes are

the living organisms, including bacteria and fungi living within plant tissues without causing any obvious pathological effects and have been found in most investigated plant species. It became obvious that endophytes are considered valuable sources of biologically active natural products, especially when several strains of the endophytic fungus *Pestalotiopsis microspora* cultured from *Taxodium distichum* (bald cypress) tree produced excellent yield of the known taxane natural anticancer drug paclitaxel (taxol®).[2] In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones.[3,4] There are several other examples highlighting the importance of endophytes as a novel resource for a variety of biologically active entities. Examples of these are: the parent of all β–lactam antibiotics penicillin G from several *Penicillium* sp., the antifungal microtubule-disrupting antibiotic griseofulvin from *Penicillium griseofulvum* fungus, the cholesterol biosynthesis inhibitor and the parent of all current statins lovastatin (monacolin K) from *Aspergillus terreus*, and many others.[5,6] Fungi species of the *Penicillium* are among the important producers of a wide range of secondary metabolites with significant pharmaceutical and therapeutic applications. Among various fermentation products of *Penicillium* species are several biologically active metabolites including ergot alkaloids, diketopiperazines, pyrrol and quinoline alkaloids.[7] *Penicillium citrinum* is a filamentous fungus belongs to the family Trichocomaceae with a worldwide distribution and considered one of the most commonly occurring fungal species on earth.[8] *P. citrinum* has been cultured from different sources such as cereals, indoor environment, and soils. The common food mycotoxin citrinin largely produced and named after *P. citrinum*. Another extrolites reported from *P. citrinum* include: quinolactacins, quinocitrinines, tanzowaic acid A, asteric acid and compactin.[9] This study reports the anti-breast cancer and antibiotic activities of different secondary metabolites from *P. citrinum* cultured from Egyptian olive tree fruits.

MATERIALS AND METHODS

General experimental procedures

Thin layer chromatography (TLC) analysis was carried on pre-coated Si gel 60 F_{254} 500 µm TLC plates (EMD Chemicals), using 1% *p*-anisaldehyde in concentrated H_2SO_4 and/or Dragendorff's reagent as chemical visualizing reagents. For column chromatography, Si gel 60 (Natland International Corporation, 230-400 µm) and Sephadex LH-20 (Sigma-Aldrich) were used with gradient CH_2Cl_2-CH_3OH mixtures as mobile phases. Generally, 1:100 ratios of mixtures to be chromatographed versus the used stationary phase were used in all liquid chromatographic purifications. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, using tetramethylsilane (TMS) as internal standard, on a JEOL Eclipse-ECS NMR spectrometer operating at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR.

Chemicals and culture media

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Organic solvents were purchased from VWR (Suwanee, GA), dried by standard procedures, packaged under nitrogen in Sure/Seal bottles and stored over 4 Å molecular sieves. Liquid compound media-α (20 g sucrose, 5 g peptone, 5 g yeast extract, 5 g NaCl, 5 g Na_2HPO_4 in 1L), solid agar media, potato dextrose agar (PDA), yeast malt extract agar (YM agar) and mycophil agar 40 g/ 1L distillated water were all used for fermentation and cultures.

Cultivation and isolation of different endophytic fungi

Olive tree organ samples (leaf, fruit, root and soil) were collected from Siwa oasis at the Egyptian western desert December 2013. Different plant parts were surface sterilized using 70 % ethanol, cultured over different solid media, including PDA, YM agar, and mycophil agar, and incubated at 28^0 C for 7 days. Ten endophytic fungal strains were successfully cultured and isolated.[10] all the steps have been done under aseptic and sterile conditions.

DNA Isolation, amplification and sequencing

Different fungal isolates grown on PDA at 28°C for 7 days were inoculated into sterile compound medium-α and incubated for 4 days in the dark at room temperature (20–21°C). On the fourth day, each fungal mass was collected and subjected to ultra-freezing using liquid nitrogen. The dried mycelia strongly triturated and crushed in a mortar into a very fine power. Microbeads were used to facilitate the mechanical disruption of fungal cell membranes, thus maximizing DNA extractability. DNA extraction followed using UltraClean™ microbial DNA isolation kit according to the manufacturer protocol (MO BIO Laboratories, Version 10062005). Extracted DNA was stored at −20°C. The resulting *g*DNA was used as a template to amplify fungal ITS-rDNA region using the primers ITS1 (5′-TCC GTA GGT GAA CCT GCG-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′).[11] The reaction mixture contained 10 µL of 5 x Phusion® HF reaction buffer (NEB), 2.5 µL of DMSO, 1 µL of 10 mM dNTPs, 0.5 µL of 50 pmol forward primer, 0.5 µl

of 50 pmol reverse primer, 1 µL of fungal g DNA, 0.5 µL of Phusion® high-fidelity DNA polymerase (2 U/µL, NEB), and 34 µL of water. PCR conditions were as follows: initial denaturation (95°C for 5 min); 30 cycles of denaturation (95°C for 45 s), primer annealing (55°C for 45 s), and elongation (72°C for 45 s), with a final elongation step at 72°C for 10 min. PCR product was purified from gel (0.8% agarose in Tris-acetate-EDTA buffer) using QIAquick®gel extraction kit (Qiagen) and used directly for sequencing using ITS1 and ITS4 primers (DNA Sequencing & Services, University of Dundee, Scotland).

***Penicillium citrinum* fermentation, extraction, and metabolites isolation**

P. citrinum isolate was grown on PDA for 7 days at 28°C after which a spore suspension was prepared and stored in 10% aqueous glycerol at −80°C for subsequent fermentation use. Initial experiments aimed at selecting the most proper media type and fermentation conditions which maximize fungal growth and metabolites production. Three media types were tested: compound medium alpha (CMα), rice media (100 g rice/1L dist. H_2O), and potato dextrose broth (40 g PD broth/1L dist. H_2O). CMα and static fermentation in dark room temperature were the best optimal fermentation conditions for this strain growth. Hence, subsequent fermentations were grown under static-dark conditions in CMα, thirty-five aluminum trays were prepared, each contained 800 mL media and fermented for 7 days at room temperature (20–21°C) in the dark. At the end of the incubation period, mycelia were harvested and cell-free liquid media collected and extracted twice with 20 L EtOAc. Extracts were collected and dried under reduced pressure to give 15 g syrupy extract. The extract was subjected to CC over Si gel 60 gradient elution, CH_2Cl_2–CH_3OH, starting with 100% CH_2Cl_2 then 5%, 10/%, 20%, 30%, and 40% to afford five fractions (I-V). Purification of different secondary metabolites from *P. citrinum* culture media extract was performed as shown in the flow chart (Figure 1).

***In vitro* assays**

Cell lines and culture conditions

The human breast cancer cell lines BT-474 (Luminal B/ER^+, $PR^{+/-}$, $HER2^+$), MDA-MB-231 (ER^-, PR^-, $HER2^-$), and MDA-MB-468 (Basal/ER^-, PR^-, $HER2^-$) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were maintained in RPMI-1640 (GIBCO-Invitrogen, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 µg/mL streptomycin and 2 mmol/L glutamine in a humidified atmosphere of 5% CO_2 at 37°C.[12] For sub-culturing, cells were rinsed twice with sterile Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS) and incubated in 0.25% trypsin containing 0.025% EDTA in PBS for 10 min at 37 ºC.[25] The released cells were centrifuged, re-suspended in fresh media and counted using hemocytometer. All cells were maintained at 37°C in an environment of 95% air and 5% CO_2 in humidified incubator. A stock solution was prepared by dissolving each tested analog in sterilized DMSO at a concentration of 20 mM for all assays and stored at 4^o C. Working solutions at their final concentrations for each assay were prepared inappropriate culture medium immediately prior to use. The vehicle (DMSO) control was prepared by adding the maximum volume of DMSO, used in preparing test compounds, to the appropriate media type such that the final DMSO concentration was maintained as the same in all treatment groups within a given experiment and never exceeded 0.1%.[12]

Measurement of viable cell number

Viable cell count was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The optical density of each sample was measured at 570 nm on a microplate reader (BioTek, VT). The number of cells per well was calculated against a standard curve prepared at the start of each experiment by plating various concentrations of cells (1,000-60,000 cells per well), as determined using a hemocytometer.[13]

MTT proliferation assay

MDA-MB-231,MDA-MB-468, and BT 474, in exponential growth, were plated at a density of 1×10^4 cells per well (6 wells/group) in 96-well culture plates and maintained in RPMI-1640 media supplemented with 10% FBS and allowed to adhere overnight at 37°C under 5% CO_2 in a humidified incubator. The next day, cells were washed with PBS, divided into different treatment groups and then fed serum-free defined RPMI-1640 media containing 40 ng/mL of HGF as a mitogen and experimental treatments (containing various doses of the specific tested compound) or vehicle-treated control media and incubation resumed at 37°C under 5% CO_2 for 72 h. Cells in all groups were fed fresh treatment media every other day during the 72 h treatment period. Control and treatment media were then removed, replaced with fresh media, and 50 µL MTT solutions (1 mg/mL) was added to each well and plates were re-incubated for 4 h. At the end of the incubation period, the color reaction was stopped by removing the media and adding 100 µL DMSO to dissolve the formazan crystals formed. Incubation at 37°C was resumed for up to 20 minutes to ensure complete dissolution of crystals. Absorbance was determined at λ 570 nm using an ELISA plate reader (BioTek, VT, USA). The IC_{50} value for each compound

was calculated by nonlinear regression (curve fit) of log (concentration) versus the % survival, implemented in GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). The % cell survival was calculated as follows: % cell survival = (Cell No. treatment/Cell No. control) × 100%.[12]

Cell migration assay

Wound-healing assay was conducted as previously described.[12,14] The confluent monolayer MDA-MB-231 cells were harvested and seeded onto a sterile 24-well plate then allowed to recover overnight. A scratch wound was inflicted using a sterile 200 µL pipette tip. Wounds were photographed on an inverted microscope connected to vista vision camera to measure the boundary of the wound at pre-migration. Subsequently, cells were treated with different concentrations of test compound in serum starved media, each in triplicate, using DMSO as vehicle control. Incubation was carried out for 20-24 h till wound just about to close in control wells. Media was then aspirated and cells were fixed by methanol prior to staining with Giemsa stain. Images were captured for each wound (three images/well) and processed by camera program (Liss View software) where the healed wound width was measured. Percent migration was calculated using the following equation:

$$\% \text{ Migration} = \left[\frac{\text{Wound width at zero time} - \text{Wound width in treaed well}}{\text{Wound width at zero time} - \text{Wound width in DMSO}}\right] \text{X } 100$$

The IC_{50} value for each compound was calculated by nonlinear regression of log concentration versus the % migration.

Antimicrobial study

Micro-organisms were obtained from NCCLS (*Bacillus subtilis* [G^+] ATCC 6051, *Staphylococcus aureus* [G^+] ATCC 12600, *Escherichia coli* [G^-] ATCC 11775, *Pseudomonas aeruginosa* [G^-] ATCC 10145, *Candida albicans* ATCC 7102 and *Aspergillus flavus* were used to test the antimicrobial activity of **1**-**3**. The antimicrobial study of the tested compounds was determined by applying modified disc diffusion method.[15] The tested compounds residue was diluted with DMSO in a ratio 1:5 w/v, then 20 µL was aseptically transferred onto sterile discs of Whatmann filter paper (5 mm diameter). Standard discs of tetracycline and amphotericin B served as positive controls for antimicrobial activity but filter discs impregnated with 20 µL of solvent (DMSO) were used as a negative control. The diameters of the inhibition zones were measured in millimeters by the use of slipping calipers (Table 6).

RESULTS AND DISCUSSION

Cultivation, extraction and isolation of fungal metabolites

Culturing of different olive tree organs (leaf, fruit, and root) and saprophytic soil, associated with the tree root, collected from Siwa oasis at the Egyptian western desert in December 2013.After surface sterilization, each organ surface and inner contents were inoculated onto three different solid media and incubated for 7 days. 1% chloramephenicol was occasionally added to the media to suppress bacterial growth. Cultures afforded ten different endophytic fungi (MS1-MS10) based on differences in their colony, mycelia, and spores morphological features (Figure 2), MS1-MS5 from root samples, MS6-MS7 from leaf samples and MS8-MS9 from fruit samples. Each fungus was cultured in CMα under both static and rotation (50-100 rpm) conditions and secondary metabolites production was monitored with TLC, ^1H, and ^{13}C NMR in order to reach the optimum culture conditions for each fungus in both media and mycelia separately for each fungus. Preliminary screening for antiproliferative activity used the breast cancer cell line MDA-MB-231 using MTT assay for all obtained extracts (media and mycelia). Results of these screening assays showed that four out of the ten isolated strain extracts (MS-2, MS-3, MS-7 and MS-9) showed significant antiproliferative activities (Table 1).

Table 1. Antiproliferative activities of different fungal strain (MS 1-10) extracts and their IC_{50} against MDA-MB-231 breast cancer cell line in MTT assay.

Endophytic strain	IC_{50} of media extract	IC_{50} of mycelia extract
MS-1	>200 µg/mL	>200 µg/mL
MS-2	>96.5 µg/mL	~182.56 µg/mL
MS-3	~103.3 µg/mL	>144.8 µg/mL
MS-4	>200 µg/mL	>200 µg/mL
MS-5	~154.3 µg/mL	>200 µg/mL
MS-6	>200 µg/mL	>200 µg/mL
MS-7	~ 84.3 µg/mL	~ 43.84 µg/mL
MS-8	~ 162.3 µg/mL	>200 µg/Ml
MS-9	~ 87.17 µg/mL	~ 110.2 µg/Ml
MS-10	>200 µg/mL	>200 µg/Ml

DNA Isolation, amplification and taxonomic identification

Phenotypic and 18S-rDNA sequence analyses, morphological and chemotaxonomic investigations were used for taxonomic identification. The phylogeny of the isolated endophytic fungus was inferred by sequence comparison of its amplified internal transcribed spacer (ITS) with published sequences obtained from GenBank.[16,17] The most related sequences were aligned, and a neighbor-joining tree was

calculated. The isolated endophytic fungi were identified and listed in Table 2.

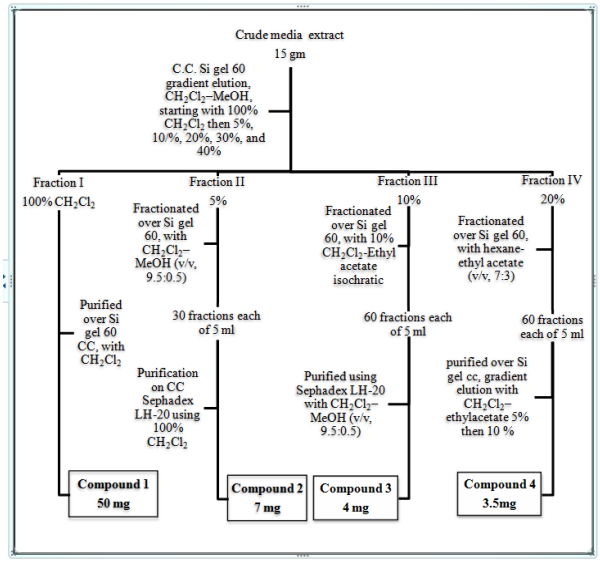

Figure 1. Flow chart of fractionation and purification of secondary metabolites from *P. citrinum*fermentation broth extract.

Table 2. Taxonomic identification of some isolated endophytic fungi.

Strain code	Endophytic fungi
MS-2	*Fusarium solani*
MS-3	*Aspergillus tubingensis*
MS-7	*Penicilliumchrysogenum*
MS-9	*Penicilliumcitrinum*

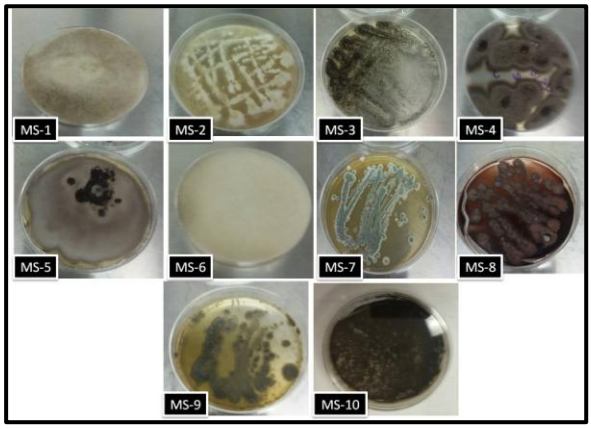

Figure 2. Different endophytic fungal strains MS1-MS10 cultured on different solid agar media isolated from Egyptian olive tree organ and saprophytic samples.

Fermentation, extraction and isolation of *P. citrinum* metabolites

After large-scale fermentation of *P. citrinum* (Figure 3), it has been noticed that EtOAc extract of the fermentation broth showed significant antiproliferative activity (IC_{50} value of 87.17 µg/mL) against the human metastatic breast cancer cell line MDA-MB-231 in MTT assay. The extract was subjected to CC Si gel 60 gradient elution, with CH_2Cl_2–CH_3OH, starting with 100% CH_2Cl_2 then 5%, 10/%, 20%, 30%, and 40% to afford five fractions (I-V). Biological evaluation of these fractions showed diverse antiproliferative activities against MDA-MB-231 cells. These activities were as follows: Fraction I (IC_{50}= 50.3 µg/mL), fraction II (IC_{50}= 41.7 µg/mL), fraction III (IC_{50}= 35.2 µg/mL), fraction IV (IC_{50} = 19.3 µg/mL) and fractions V (IC_{50} > 40 µg/mL). These fractions were subjected to further purification using different chromatographic procedures to yield four known compounds; citrinin (**1**), 2-(hept-5-enyl)-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4*H* pyrrolo [2,1-*b*]-1,3-oxazine (**2**), scalusamide A (**3**), and perinadine A (**4**).

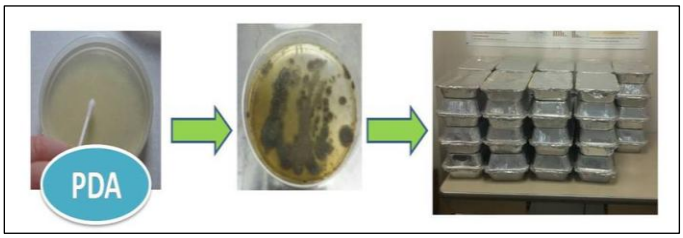

Figure 3. Overview of *Penicillium citrinum* large-scale fermentation methodology.

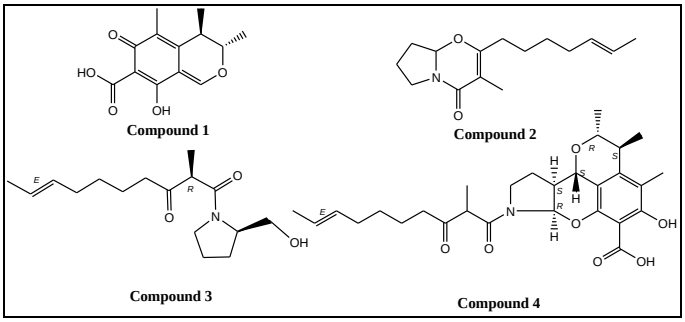

Figure 4. Structure of compounds isolated from *Penicillium citrinum* Culture media extract

Characterization and identification of isolated compounds

The isolated compounds were identified based on their mass spectrometry and 1D and 2D NMR spectroscopic analysis which coincided with the literature.[18-21]

Compound **1** (50 mg) showed a UV active spot on pr-coated Si gel 60 TLC at 254 nm with no change

upon spraying with *p*-anisaldhyde/H_2SO_4. The ^{1}H NMR spectrum of **1**showed three signals corresponding to three methyl groups appearing as one singlet signals at δ 1.99 (3H, s) H-11, and two methyl doublets at δ 1.2 (3H, d, *J*=6.88) and 1.32 (3H, d, *J*=6.44) assigned for H-9 and H-10, respectively, along with two significant multiplet signals at δ 2.97 (1H, m) and 4.77 (1H, m) assigned for protons H-4 and H-3, respectively, in addition to a singlet signal at δ 8.22 assigned for the oxygenated olefinic proton H-1. The ^{13}C PENDANT NMR spectrum showed thirteen carbon signals, including carbon signals for three methyl groups at δ 18.6, 18.6, and 18.3 assigned for methyl groups C-9, C-10, and C-11. In addition to a downfield carbon signal for an oxygenated olefinic carbon at δ 162.8 assigned for C-1, beside two significant methine signals at δ 34.7 and 81.7 assigned for carbons C-4 and C-3, respectively. In addition, seven quaternary carbons, including the C-12 carboxylic acid carbonyl carbon at δ 174.6, were observed. The structure of compound **1** was also confirmed through 2D NMR spectroscopy experiment HMQC and HMBC correlations (Table 3) and comparison with reported data.[18] Thus compound **1**was proved to be citrinin.

Compound **2** (7 mg) showed a UV active spot, λ_{254}, on Si gel 60 TLC without distinct color with *p*-anisaldhyde/H_2SO_4 but showed an orange spot with Dragendorffs reagent. Its ^{1}H NMR spectrum showed signals corresponding a methyl singlet signal at δ 1.78 (3H, s) assigned for H_3-1``, and abroad methyl doublet at δ 1.61 assigned for H-7` along with two significant multiplet signals at $\delta$ 5.38 (1H, m) and 5.36 (1H, m) assigned for the two olifenic protons H-5` and H-6`, respectively, in addition to a downfield multiplet H-8a signal at δ 5.19 (1H, m) which is downfield shifted due to its nearby nitrogen and oxygen atoms. The ^{13}C PENDANT NMR spectrum of **2** showed fifteen carbon signals including two methyl carbons at δ 18.04 and 10.2, assigned for C-7 and C-1``. In addition to the two downfield olefinic carbon signals at δ 131.1 and 125.2, assigned for C-5` and 6`, respectively. The additional methine carbon at δ 87.6 assigned for C-8a and the seven quaternary carbons included a carbonyl carbon at δ 163.7 C-4 of the oxazine moiety and completed the structural skeleton of **2**. Interpretations of compound **2** protons and carbons were aided by the HMQC experiment (Table 3). Comparison of NMR data of **2** with those reported in literature[19] confirmed its identity as: 2-(Hept-5-enyl)-3-methyl-4-oxo-6,7,8,8a,tetrahydro-4*H*-pyrrolo[2,1-*b*]-1,3-oxazine.

Compound **3** (4 mg) showed no UV activity on Si gel 60 TLC, no distinct color with *p*-anisaldhyde/H_2SO_4 spray reagent but showed intense orange spot with Dragendorff's reagent. The ^{1}H NMR spectrum of **3**showed two signals corresponding to two methyl groups, a doublet at δ 1.61assigned for H_3-15, and a broad doublet at δ 1.37 assigned for H_3-17.It also showed two downfield olefinic multiplets at δ 5.37 and 5.34 (1H, m), assigned for protons H-13 and H-14, respectively. The ^{1}H NMR spectrum of **3** also showed downfield shifted nitrogenated multiplet signals at δ 4.21, proton H-2, and δ 3.56, proton H-5. The ^{13}C PENDANT NMR spectrum of **3** showed sixteen carbon signals, including two methyl carbons at δ 18.02 and 13.1 assigned for C-15 and C-17 respectively. It also showed the olefinic downfield methine carbon signals at δ 130.9 and 125.3 assigned for carbons C-13 and C-14, respectively. It also showed nitrogenated methine and methylene carbon signals at δ 61.4, at δ 48.3 assigned for carbons C-2 and C-5, respectively, in addition to two quaternary carbons, a carbonyl carbon at δ 171.1 and a ketone carbon at δ 207.1, assigned for carbons C-6 and C-8, respectively. Assignment of compound **3** NMR was aided by the HMQC experiment (Table 4) and comparison with published reported data[20] and thus it was proved to be scalusamide A.

Compound **4** (3.5 mg) showed a UV active spot on Si gel 60 TLC under the short UV wave length (254 nm). It showed no distinct color with *p*-anisaldhyde/H_2SO_4 reagent but showed intense orange spot upon spraying with Dragendorff's reagent. The ^{1}H NMR spectrum of **4** showed two signals corresponding to five methyl groups: a multiplet signal at δ 1.28, H_3-9, a methyl doublet at δ 1.29 (*J*=6.8) assigned for H_3-10, a singlet signal at δ 2.17 assigned for H_3-11, a narrow doublet signal at δ 1.61 (*J*= 4.56) assigned for H_3-15`, and a doublet signal at $\delta$1.39 (*J*=6.8) assigned for $H_3$-16`.It also showed two downfield olefinic multiplet signals at δ 5.33 and 5.36, assigned as protons H-13` and H-14`, respectively, in addition to the nitrogenated downfield doublet at δ 6.27 (*J*=7.3) assigned for proton H-2`and at $\delta$ 3.51 assigned for proton H-5`. The ^{13}C PENDANT NMR spectrum of **4** showed twenty-eight carbons, include two carbon signals for five symmetric methyl groups at δ 22.9, 18.1, 10.1, 18.1 and 13.2, assigned for C-9, C-10, C-11, C-15` and C-16`, respectively. The PENDANT spectrum of **4** also contained two olefinic methine carbons at δ 131.1 and 125.1, assigned for C-13` and 14`, respectively, beside the nitrogenated methine and methylene signals at δ 88.4 and δ 46.3 assigned for carbons C-2` and C-5`. Other carbons included two quaternary carbons, including the carbonyl carbon at δ 171.6 and the ketone carbon at δ 206.5 assigned for carbons C-6` and C-8`, respectively. Assignment of NMR data of compound **4** was also aided by the 2D NMR spectroscopic experiments HMQC and HMBC (Table 4). Comparison of compound **4** NMR data with published literature[21] confirmed its identity as the known pyrrolidine alkaloid perinadine A.

Table 3. ^{1}H NMR and ^{13}C NMR spectroscopic data of compounds 1 and 2.[a]

Compound **1**			Compound **2**		
Position	δ^{13}C	δ^{1}H (multiplicity, *J* in Hz)	Position	δ^{13}C	δ^{1}H (multiplicity, *J* in Hz)
1	162.8, CH	8.22, s	1	-	-
2			2	163.6, C	-
3	81.7, CH	4.77, m	3	106.5, C	-
4	34.7, CH	2.97, m	4	163.7, C	-
4a	139.1, C	-	5	-	-
5	123.2, C	-	6	44.4, CH_2	3.55, 3.86, 2H, m
6	183.9, C	-	7	32.3, CH_2	1.94, 2H, m
7	100.4, C	-	8	31.8, CH_2	2.26, 2H, m
8	177.2, C	-	8a	87.6, CH	5.19, m
8a	107.5, C	-	1`	30.7, CH_2	2.31, 2H, m
9	18.3, CH_3	1.32 , d(6.4)	2`	29.5, CH_2	1.51, 2H, m
10	18.6, CH_3	1.21, d (6.9)	3`	26.4, CH_2	1.47, 2H, m
11	9.5, CH_3	1.99, s	4`	21.9, CH_2	2.1, 1.81, 2H, m
12	174.6, C	-	5`	131.1, CH	5.38, m
			6`	125.2, CH	5.36, m
			7`	18.04, CH_3	1.61, brd
			1``	10.2, CH_3	1.78, s

Table 4. ^{1}H NMR and ^{13}C NMR spectroscopic data of compounds 3 and 4.[a]

Compound **3**			Compound **4**		
Position	δ^{13}C	δ^{1}H (multiplicity, *J* in Hz)	Position	δ^{13}C	δ^{1}H (multiplicity, *J* in Hz)
1			1	62.3, CH	4.85 (1H, m, *J*=6.8)
2	61.4, CH	4.21 (1H, m)	3	74.3, CH	4.12 (1H,m, *J*=6.88)
3	28.2, CH_2	2.06 (2H, m)	4	34.7, CH	2.69 (1H, m)
4	24.4, CH_2	1.96 (2H, m)	4a	143.7, C	-
5	48.3, CH_2	3.56 (2H, m)	5	119.4, C	-
6	171.1, C		6	160.8, C	-
7	53.4, CH	3.55 (1H, m)	7	99.9, C	-
8	207.1, C		8	147.2, C	-
9	39.4, CH_2	2.47 (2H, m)	8a	109.5, C	-
10	23.1, CH_2	1.53 (2H, m)	9	22.9, CH_3	1.28 (2H, m)
11	29.1, CH_2	1.31 (2H, m)	10	18.1, CH_3	1.29 (3H, d, *J*=6.8)
12	32.4, CH_2	1.93 (2H,m)	11	10.1, CH_3	2.17 (3H, s)
13	130.9, CH	5.37 (1H, m)	12	171.7, C	-
14	125.3, CH	5.34 (1H, m)	2`	88.3, CH	6.27 (1H, d, *J*=7.32)
15	18.02, CH_3	1.61 (3H, d)	3`	42.2, CH	3.14 (1H, m)
16	66.6, CH_2	3.73 (2H, d)	4`	29.8, CH_2	1.41 (2H, m)
17	13.1, CH_3	1.37 (3H, br d)	5`	46.4, CH_2	3.51 (2H, m)
			6`	171.6, C	
			7`	53.8, CH	3.47 (1H, m)
			8`	206.5, C	
			9`	38.9, CH_2	2.58, (1H, m)
			10`	24.6, CH_2	1.29 (2H, m)
			11`	28.8, CH_2	1..32 (2H, m)
			12`	32.4, CH_2	1.95 (2H, m)
			13`	131.1, CH	5.33 (1H, m)
			14`	125.1, CH	5.36 (1H, m)
			15`	18.1, CH_3	1.61 (3H, br d)
			16`	13.2, CH_3	1.39 (3H, d, *J*=6.84)

[a]In $CDCl_3$, 400 MHz for ^{1}H and 100 MHz for ^{13}C experiments. Coupling constants (*J*) in Hz. Carbon multiplicities were determined by PENDANT experiment, C = quaternary, CH = methane, CH_2 = methylene and CH_3= methyl carbons.

The antiproliferative activities of compounds 1-4 were tested using MTT colorimetric assay against the human breast cancer cell lines BT-474, MDA-MB-231, and MDA-MB-468. At least four concentrations per compound were tested per cell line which was used to calculate each IC_{50} value. The olive phenolic (-)-oleocanthal with validated activity was used as a positive control at different doses (10, 20, and 40 µM) against the different cancer cell lines.12, 22 Compound 1 did not show activity against the tested cells up to 40 µM. Whereas, alkaloids 2-4 showed significant antiproliferative activity against the human metastatic breast cancer cell line MDA-MB-231 with IC_{50} values of 10.6, 14.8, and 17.7 µM, respectively (Table 5 and Figure 5).

Table 5. Antiproliferative activities of compounds 1-4 (IC_{50} µM) against human breast cancer cell lines.

Cell line	1	2	3	4
BT-474	> 40	75.8 ± 3.2	> 40	25.6 ± 4.2
MDA-MB-231	> 40	10.6 ± 1.5	14.8 ± 3.5	17.7 ± 3.1
MDA-MB-468	> 40	14.7 ± 1.2	24.9 ± 2.1	38.9 ± 2.9

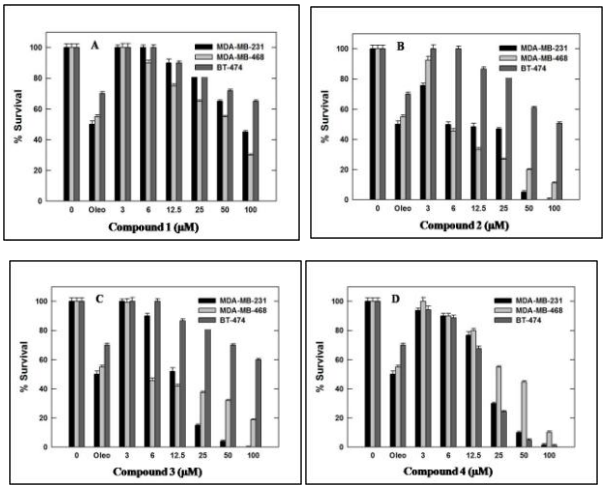

Figure 5. Compounds 1-4 inhibit HGF-induced proliferation of three different breast cancer cell lines MDA-MB-231, MDA-MB-468, and BT-474. (A) Compound 1, (B) Compound 2, (C) Compound 3, and (D) Compound 4. Error bars indicate the SEM of *n*=3/dose. Oleocanthal was used as a positive control.[12,22]

Cell migration assay

HGF/c-Met pathway dysregulation is known to activate migration and invasion of c-Met-dependent malignancies. To assess the effect of compounds **1-4** on the HGF-induced MDA-MB-231 cells migration, the wound healing assay was performed. A 40 ng/mL dose of HGF significantly induced cellular migration with more than 85% wound closure after a 24 h treatment period. Figure 6B shows the ability of perinadine A **(4)** to significantly suppress the HGF-induced cell migration in a dose dependent manner. Treatment of cells with 2.5, 5, 7.5, 10, 15 and 20µM of perinadine A for 24 h significantly inhibited cell migration in a dose-responsive manner, with an IC_{50} value of 9.7 µM (Figure 6A). Compounds **1-3** did not show significant antimigratory activity.

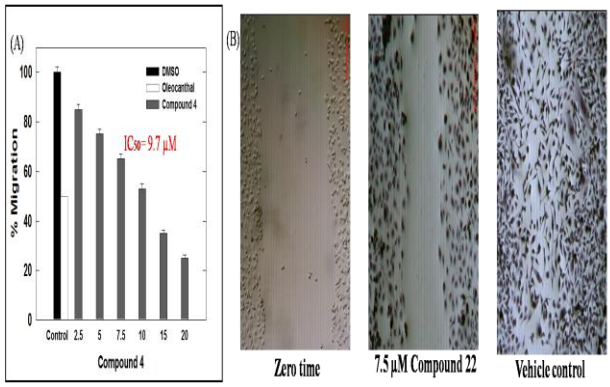

Figure 6. A. Effect of compound 4 treatment on MDA-MB-231 cell migration in wound healing assay at different concentrations compared to DMSO as vehicle control. B. Antimigratory activity of compound 4 against MDA-MB-231 breast cancer cells in WHA comparing DMSO vehicle treatment control with 7.5 µM treatment of compound 4. Error bars indicate the SEM of *n*=3/dose. (-)-Oleaocanthal was used as a positive control at 10 µM.[12,22]

Antimicrobial activity

The agar diffusion test (Kirby–Bauer antibiotic testing) was used to test the microbial sensitivity to experimental antimicrobial agents. Out of the tested compounds, citrinin (**1**) exerted marked antibiotic activity, inhibition zone diameter, against tested Gram (-) and Gram (+) bacteria up to several-fold better than the used tetracycline positive control (Table 6 and Figure 7). Compounds **2-3** exerted minimal anti-microbial activity against the tested bacteria. All compounds did not show significant antifungal activity.

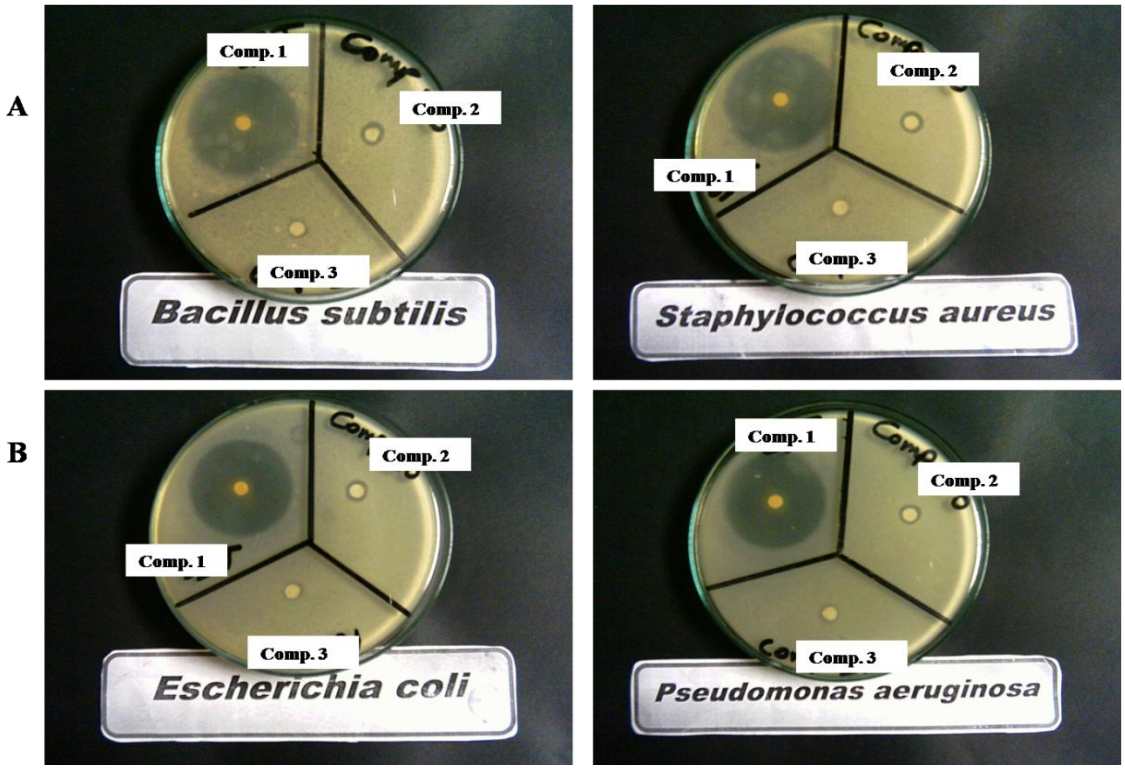

Figure 7. Antimicrobial activity using paper disc diffusion methods. A. The inhibition zone of compounds 1-3 against Gram (+) bacteria. B. The inhibition zone of compounds 1-3 against Gram (-) bacteria

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Table.6. Antimicrobial screening of compounds 1-3.

Sample		Inhibition zone diameter (mm/mg sample)					
		Bacterial species				Fungi	
		Gram (+)		Gram (-)			
		Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Aspergillus flavus*	*Candida albicans*
DMSO Control		0.0	0.0	0.0	0.0	0.0	0.0
Standard	Tetracycline	26	21	25	26	--	--
	Amphotericin B	--	--	--	--	17	19
Compound 1		41	43	41	40	0.0	10
Compound 2		12	12	12	11	0.0	0.0
Compound 3		10	9	9	9	0.0	0.0

CONCLUSION

The Egyptian-based olive tree afforded multiple endophytes with diverse chemical and bioactivity profiles. Bioassay-guided fractionation of the endophytic fungus *P. citrinum* afforded the polyketide citrinin (**1**) and 3 known pyrrolidine alkaloids (2-(hept-5-enyl)-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4*H*-pyrrolo[2,1-*b*]-1,3-oxazine (**2**), scalusamide A (**3**) and perinadine A (**4**). Perinadine A **(4)** exhibited significant anti-proliferative activity against the human breast cancer cell lines MDA-MB-231, MDA-MB-468, and BT-474, with IC_{50} values of 17.7, 38.9 and 25.6 µM, respectively. Perinadine A also exhibited significant migratory inhibition activities against MDA-MB-231 cells, with an IC_{50} value of 9.7µM. Citrinin (**1**) showed potent antibiotic activity against Gram (+) and Gram (-) bacteria and did not show significant antifungal activity. Perinadine A and citrinin are novel scaffold entities appropriate for future development to control breast malignancies and infectious diseases.

Abbreviations:

***HGF**: Hepatocyte Growth Factor, **HER2**: Human Epidermal Growth Factor Rece; ptor 2, **MTT**: 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide, **WHA**: Wound-Healing Assay, **ER-α**: Estrogen Receptor Alpha, **PR$^{+/-}$**: Progesterone-receptor-positive/negative, **YM** agar: Yeast Malt extract agar, **PDA**: Potato dextrose agar, **CMα**: Compound media α, **DNA**: Deoxynucleic acid, **ITS**: Internal Transcribed Spacer, **EDTA** : Ethylenediaminetetraacetic acid, **FBS**: Fetal Bovine Serum, **PBS**: Phosphate Buffer Saline, **RPMI:** Roswell Park Memorial Institute (RPMI) 1640, **ELISA**: Enzyme-linked immunosorbent assay, **IC_{50}**: Half maximal inhibitory concentration, **NCCLS**: National Committee for Clinical Laboratory Standards*

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Conflict of Interest:

The authors declare that they don't have any conflict of interest.

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