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# Anti-HCV protease potential of endophytic fungi and cytotoxic activity

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ABSTRACT

Forty-eight endophytic fungal strains were isolated and purified from ten Egyptian medicinal plants and their culture broth extracts were explored for HCV protease inhibitory activity and cytotoxicity. The ethyl acetate extracts of *Alternaria alternata* PGL-3, *Cochlibolus lunatus* PML-17, *Nigrospora sphaerica* EPS-38, followed by *Emerecilla nidulans* RPL-21 showed the most potent inhibition of HCV NS3/4A protease with $IC_{50}$ 17.0, 20.5, 33.6, and 54.6 μg/ml, respectively, with low cytotoxicity except for the later. The extracts of *Emericella nidulans* RSL-24, *Fusarium oxysporum* SML-41, *Emericella nidulans* RPL-21, and *Penicillium* sp. RSL-43 exhibited strong cytotoxic activity against human breast cancer cell lines (MCF-7) with $IC_{50}$ 10.8, 11.0, 12.5, and 13.7 μg/ml, respectively. *Emericella nidulans* RSS-22, *Emericella nidulans* RSL-24, and *Fusarium oxysporum* SML-41 displayed a potent cytotoxic effect on human liver cancer cell lines (HEP-G2) with $IC_{50}$14.8, 20.3 and 24.0 μg/ml, respectively. Alternariol and alternariol -9-methyl ether were isolated from the ethyl acetate extract of *Alternaria alternata* PGL-3 whereas, emericellin, shamixanthone, arugosin C were isolated from the ethyl acetate extract of *Emericella nidulans* RPL-21. The results suggest *Alternaria alternata* PGL-3 endophyte from *Punica granatum* peel as a source of antiviral lead.

## 1. Introduction

Hepatitis C virus (HCV) infection is a growing public health problem. Globally, an estimated 71 million people have chronic hepatitis C, who often will develop cirrhosis or liver cancer (WHO 2018). The number of patients with serious liver hepatocellular carcinoma resulting from chronic HCV infection is projected to increase, the primary reason for liver transplantation (Kim et al., 2013). Despite research in this area is ongoing up till now, there is no vaccine for hepatitis C (WHO 2018). Recently, the treatment depends mainly on direct-acting antiviral agents where HCV NS3/4A protease is considered as a key target of efforts to develop antiviral inhibitors (De Francesco and Carfi, 2007). In addition, like antibiotics, the emergence of cross-resistance urges the sustainable discovering of novel molecules or modulation of the present one.

Recently, drug discovery research has shown considerable interest in plant derived microorganisms, such as endophytic fungi, as sources of biologically active molecules. Endophytic fungi reside in the plant's internal tissues without causing apparent diseases symptoms. Some of these endophytes provide protection to their host plant by producing secondary metabolites that are involved the host plant's defense mechanism (Hyde and Soytong, 2008; Krings et al., 2012; Purahong and Hyde, 2011). Endophytic fungi received much attention after the detection of taxol as anti-cancer drug from the endophytic fungus *Taxomyces andreanae* that had been isolated from *Taxus brevifolia* plant (Stierle et al., 1993, 1995).

Medicinal plants around the world appear to harbor high biodiversity of endophytic microbes, including many fungal species, which are potential producers of bioactive molecules (Huang et al., 2008; Phongpaichit et al., 2008; Rosa et al., 2010). According to Strobel (2002), the plant selection should be governed by reasonable protocols for the discovery of bioactive endophytic fungi; this includes examining plants reputed for medicinal or pharmacological activities. Thus, the Egyptian plant species in this study were selected on the basis of their

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**Table 1**
Voucher numbers of collected plant species.

| Plant species | Voucher | Plant species | Voucher |
|---|---|---|---|
| *Euphorbia pulcherrima* | 000099 CE 04-08-06-12 | *Punica granatum* | 000220 CP 06-02-03-01 |
| *Plantago major* | 000152 ww 08-06-01-06 | *Solanum nigrum* | 000195 ww 09-02-02-05 |
| *Silybum marianum* | 000184 ww 08-07-01-01 | *Salvadora persica* | 000301 CS 03-03-06-07 |
| *Ruprechtia salicifolia* | 000107 CR 06-03-05-02 | *Phoenix dactylifera* | 000037 CP 06-05-02-10 |
| *Ruprechtia polystachya* | 000107 CR 06-03-05-012 | *Synadenium grantia* | 000112 CS 09-02-05-04 |

potential therapeutic properties and traditional medicine. For example, *Silybum marianum* is cultivated in Egypt and recommended in traditional European and Asiatic medicine for liver disorders treatment (Morazzoni and Bombardelli, 1995). Silymarin is the active principle in *S. marianum* which is an important free radical scavenger that protects human hepatic tissues from oxidative damage (Abbasi et al., 2010). Also, *Plantago major* is a popular wound healing herb with antiviral activity (Haddadian et al., 2014; Chiang et al., 2003) while its seeds have gastroprotective and hepatoprotective effects (Najafian et al., 2018). Furthermore, *Punica granatum* is used in various systems of medicine and its potential therapeutic applications are wide-ranging including antimicrobial and anticancer (Miguel et al., 2010; Howell and D'Souza, 2013). Although some species of the family Euphorbiaceae produce latex are toxic, *Euphorbia pulcherrima* is not toxic where the plant is used as a concoction in folk medicine for treatment of ailments such as resembling typhoid and gastro-enteritis in Kano Nigeria (Phongpaichit et al., 2008). Moreover, some *Ruprechtia* species are widespread in Egypt, exhibited immunomodulatory, anti-inflammatory, anticancer and antibacterial activities as well as inhibition on glucose-6-phosphatase system (Bruzual De Abreu et al., 2011; Haggag et al., 2013).

However, there are few reports on endophytic fungi from the selected plants. These include some fungal species from inner tissues of *Plantago lanceolata* leaves (Rosa et al., 2010), from the stems, leaves, roots, and seeds of *Silybum marianum* (Rosa et al., 2010; El-Elimat et al., 2014; Figueroa et al., 2014), *Penicillium glabrum* from pomegranate fruits of *Punica granatum* (Naik et al., 2008) and *Fusarium* sp. was isolated from *Euphorbia* sp. (Dai et al., 2008). Thus, in this study, we aimed to isolate and identify the endophytic fungi from ten Egyptian medicinal plants. The culture broth extracts of the isolated fungi were evaluated for their inhibitory effect on HCV NS3/4A protease using a SensoLyteTM 520 HCV protease assay kit as well as their cytotoxic activity. In addition, the isolation and structure elucidation of the chemical constituents of the bioactive extracts were carried out.

## 2. Materials and methods

### 2.1. General experimental

Culture media: Glucose, (Acros Organics), bacteriological peptone, malt extract, yeast extract powder and nutrient agar (Lab M Limited), $K_2HPO_4$ (Laboratory Reagent), $MgSO_4$ (Oxford Laboratory Reagent), agar (Sisco Research Laboratories Pvt. Ltd). Hepatitis Virus C NS3 protease inhibitor 2 (cat#AS-25346), Sensolyte TM 520 HCV Protease assay kit Fluorimetric (cat#AS-71145), HCV NS3/4A protease and Sensolyte TM Green Protease Assay Kit Fluorimetric (cat#AS-71124) were purchased from AnaSpec Inc., San Jose, Ca, USA. Chymotrypsin inhibitor (Soybean trypsin) was purchased from Sigma Aldrich Co., and Becton Dickinson Falcon TM Microtest 384-well 120 µL black assay plates, no lid, non-sterile, was purchased from Becton Dickinson Inc, Franklin Lakes. NMR measurements were carried out using Bruker 600 spectrometer (Bruker Biospin, Rheinstetten, Germany). Electron ionization mass spectrometry was performed using Finnigan-MatSSQ 7000 spectrometer (Thermo Fisher Scientific, Waltham, MA USA 02451). Silica gel 60 (70–230mesh; E. Merck, Darmstadt, Germany) and Sephadex LH-20 CC (Pharmazia, Sweden) for column chromatography (CC). Silica gel plates 60F $_{254}$ (Merck, Darmstadt, Germany). Solvents used for plant extraction were from SDFCL (Industrial Estate, 248 Worli Road, Mumbai-30, India).

### 2.2. Host samples collection

Ten host samples of Egyptian plants were collected from different areas in Cairo, Egypt. The samples of stem, leaf, and root from the selected plants were harvested on the basis of a clean and healthy exterior. The collected specimens were washed by distilled water and processed freshly for fungal isolation. Voucher specimens were identified by Dr. Mohamed El-Gebaly, Cairo University. The voucher specimens have been deposited at the herbarium of the Al-Orman garden, Giza, Egypt (Table 1).

### 2.3. Fungal isolation and culture conditions

A series of surface sterilization techniques were done for each host plant sample (stem, leaf and root) to isolate truly plant endophytic fungal strains. Once the host was collected, the fresh samples were thoroughly surface washed with 70% ethanol three times with sterile distilled water to remove the residue of ethanol. Small pieces of inner tissue were rinsed with sterile water then cut into small segments. These segments of each sample carefully excised and placed on different isolation media containing antibiotic. After 7 days of incubation, hyphal tips of the fungi are removed and transferred to potato dextrose agar (PDA) plates. Fungal colonies were transferred to one or more of the media for purification and identification. Each fungus was isolated as an epiphyte using appropriate media at 28 °C. The pH 7.5 has been adjusted in water supplemented with penicillin benzyl sodium salt (0.02) to avoid any bacterial growth. After 7–10 days, velvety colonies were observed.

### 2.4. Morphological identification of the fungal isolates

Forty-eight endophytic fungal strains were isolated from the collected medicinal plant samples. Purification of the isolated strains was performed by successively agar culture technique. The pure isolated fungal strains were identified mainly by morphological methods that have been performed by scrutinizing the culture, the mechanism of spore production, and the characteristics of the spores by macroscopic (colony morphology, texture, color, shape, and size) and microscopic examination. For inducing sporulation, each of the isolated strain was separately inoculated on agar Petri dishes. All experiments and observations were repeated twice. All isolated fungal strains were fully morphologically identified. The identification of two selective bioactive strains was subjected to further 18S rDNA sequence comparison.

### 2.5. Molecular identification of the endophytic isolates

The Nucleic acid was extracted from isolated endophytic purified using the GenEluteTM DNA isolation kit for genomic DNA (Sigma Aldrich) using the Chomczynski method (Chomczynski and Sacchi, 1987). For identification and differentiation, the Internal Transcript Spacer regions (ITS1 and ITS4) and the intervening 5.8S rRNA region was amplified and sequenced using electrophoretic sequencing on

3130-genetic analyzer (Fermentas company: taq polymerase, dntps) using GenJETTM sequencing kit. The DNA fragment of the ITS regions was amplified using polymerase chain reaction (PCR) with the pair of primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), by the reaction system and thermocycler parameters and the assay of the PCR products was performed by the method of Hall (1999).

### 2.6. Extraction and isolation of chemical metabolites

The fifteen days old fermentation broth (12–20 L) was separated from the fungal mat. The fungal mycelia were suspended in distilled water for easy blending by an Ultra Turrax Model 25 at 8000 rpm, and the homogenate was extracted by ethyl acetate. The resultant extract was evaporated to dryness using a rotavapor at 40C, followed by defatting with *n*-hexane. The bioactive crude extract *A. alternata* PGL-3 *alternate* and *E. nidulans* RPL-21 were individually chromatographed over a silica gel column using *n*-hexane as starting nonpolar eluent and gradually increasing the polarity using ethyl acetate as polar solvent in the eluent mixture (5%, 10%, until 100% ethyl acetate), followed by 20% and 50% methanol–ethyl acetate. The combined fractions are further purified using preparative TLC using solvent system *n*-hexan/ethylacetate and Sephadex LH-20 where compounds **1–2** were isolated from *A. alternata* PGL-3 and compounds **3–5** were isolated from *E. nidulans* RPL-21. The pure compounds were subjected to analysis by MS and NMR spectroscopy.

*Alternariol* (**1**): white powder (19 mg); $^{1}$H NMR($CDCl_3$): δ7.19 (1H, br, H-10), 6.65 (1H, d, $J$ = 1.5, H-2), 6.56 (1H, d, $J$ = 1.5, H-4), 6.32 (1H,br, H-8), 2.70 (3H, s, 1-CH3); 13C δ 167.6 (C-9), 167.0 (C-9) 166.3 (C-7), 160.0 (C-3), 154.59 (C-4a), 140.13 (C-1),139.9 (C-10b), 118.7 (C-2), 111.0 (C-10b), 105.8 (C-10), 102.9 (C-1), 102.2 (C-8), 95.7 (C-6a). The spectral data were in accordance with the literature reported (Gu, 2009).

*Alternariol-9-methyl ether* (**2**)*:* white powder (14 mg); $^{1}$H NMR ($CDCl_3$): δ7.17 (1H, br, H-10), 6.69 (1H, br, H-2), 6.61 (1H, d, $J$ = 1.5 Hz, H-4), 6.58 (1H, d, $J$ = 1.5 Hz, H-8), 3.92 (1H, s, 9-$OCH_3$), 2.69 (1H, s, 1-$CH_3$). 13C NMR ($CDCl_3$): δ166.0 (C-9), 164.6 (C-6), 164.1 (C-7), 158.6 (C-3), 152.5 (C-4a), 138.3 (C −1), 137.7 (C-10a),117.5 (C-2), 108.7 (C-10b),103.3 (C-10), 101.5 (C-4), 99.1 (C-8), 98.3 (C-6a), 55.7(9-OCH3), 25 (1-$CH_3$). ESI MS: m/z 273 (100%) [M + H]+, 295 [M + Na]+. The spectral data were in accordance with the literature reported (Gu, 2009).

*Emericellin* (**3**): Yellow needle crystals s (22 mg). $^{1}$H NMR ($CDCl_3$): δ 12.56 (1H, s, 1-OH), 7.47 (1H, d, $J$ = 8.5 Hz, H-3), 7.35 (1H, s, H-5), 6.77 (1H, d, $J$ = 8.5 Hz, H-2), 5.65 (1H, 2dd, $J$ = 1.3 &3.0 Hz, H-2′), 5.34 (1H, 2dd, $J$ = 1.3 &4.6 Hz, H-2″), 5.11 (2H, s, 11-$CH_2$), 4.48 (2H, d, $J$ = 7.2 Hz, 1′-$CH_2$), 3.52 (2H, d, $J$ = 7.2 Hz, 1″-$CH_2$), 2.49 (3H, s, 6-$CH_3$), 1.84 (3H, s, 4″-$CH_3$), 1.81 (3H, s, 4′-$CH_3$), 1.78 (3H, s, 5′-$CH_3$), 1.75 (3H, s, 5″-$CH_3$); $^{13}$C NMR ($CDCl_3$): δ 184.6 (C-9), 159.9 (C-1), 154.0 (C-7), 152.8 (C-4a), 152.6 (C-10a), 142.6 (C-6), 139.1 (C-8), 136.9 (C-3), 134.2 (C-3′), 133.4 (C-3″), 121.6 (C-2″), 119.6 (C-2′), 119.4 (C-5), 118.9 (C-4), 117.9 (C-8a), 110.0 (C-2), 109.0 (C-9a), 72.2 (C-1′), 57.1 (C-11), 27.4 (C-1″), 25.8 (C-4″), 25.7 (C-4′), 18.1 (C-5′), 17.9 (C-5″), 17.7 (6-$CH_3$). ESI-MS: *m/z* 409 $[M+H]^+$. The spectral data were in good agreement with the literature reported (Pornpakakul et al., 2006).

*Shamixanthone* (**4**): Yellow needle crystals (25 mg). $^{1}$H NMR ($CDCl_3$): δ 12.62 (1H, s, 1-OH), 7.46 (1H, d, $J$ = 8.4 Hz, H-3), 7.32 (1H, d, $J$ = 5.4 Hz, H-5), 6.76 (1H, d, $J$ = 8.4 Hz, H-2), 5.43 (1H, d, $J$ = 1.8 Hz, H-11), 5.33 (1H, m, H-18), 4.82 (1H, s, H-15), 4.61 (1H, s, H-15a), 4.45 (1H,dd, $J$ = 1.2&3.0 Hz, H-13), 4.36 (1H, d, $J$ = 3.0 Hz, H-13a), 3.52 (2H, m, H-17), 2.83 (3H, s, 6-$CH_3$), 2.75 (1H, d, $J$ = 8.4 Hz, H-12), 1.87 (3H, s, H-16), 1.81 (3H, s, H-21), 1.77 (3H, s, H-20); $^{13}$C NMR ($CDCl_3$): δ184.5(C-9), 159.7 (C-1), 152.8 (C-4a), 152.2 (C-10a), 149.4 (C-7), 142.6 (C-14), 138.3 (C-6), 136.5 (C-3), 133.2 (C-19), 121.6 (C-18), 120.9 (C-8), 119.3(C-5), 118.9 (C-4), 116.9 (C-8a), 112.2 (C-15), 109.7 (C-2), 109.2 (C-9a), 17.4 (6-$CH_3$), 63.2 (C-11), 64.5 (C-13), 44.9 (C-12), 27.5 (C-17), 25.7 (C-20), 22.5 (C-16), 17.9(C-21). ElMS m/z: 406 The spectral data were in good agreement with the literature reported (Pornpakakul et al., 2006).

*Arugosin C* (**5**): Yellow amorphous (18 mg). $^{1}$H NMR($CDCl_3$): δ13.85 (1H, s, 3-OH), 10.71 (1H, s, 13-OH), 7.3 (1H, d, $J$ = 8.4 Hz, H-5), 6.84 (1H, s, H-12), 6.42 (1H, d, $J$ = 8.4 Hz, H-6), 5.33 (1H, t, $J$ = 8.4 Hz, H-16), 5.09 (1H, d, $J$ = 4.2 Hz, H-8), 4.39 (1H, dd, $J$ = 4.2 & 3.6 Hz, H-20), 4.21 (1H, dd, $J$ = 6.6&7.2 Hz, H-20a),3.33 (2H, d, $J$ = 8.4 Hz, H-15),2.38 (1H, q, $J$ = 4.2 Hz, H-21),2.26 (3H, 5, (11-$CH_3$), 1.78 (6H, s, H-18 &19), 1.35 (3H, s, H-24), 1.28 (3H, s, H-23); 13C NMR ($CDCl_3$): δ197.1 (C-1), 112.5 (C-2), 163.4 (C-3), 124 (C-4), 127.7 (C-5), 108.8 (C-6), 159.0 (C-7), 74.1 (C-8), 120.5 (C-9), 145.2 (C-10), 136.4 (C-11), 16.5 (11-$CH_3$), 120.2 (C-12), 155.6 (C-13), 119.6 (C-14), 27.7 (C-15), 121.8 (C-16), 133.2 (C-17), 17.8 (C-18), 25.8 (C-19),65.1(C-20), 49.3 (C-21), 71.1 (C-22), 28.9 (C-23), 28.2 (C-24). ESI-MS: m/z 425 [M + H] +. The spectral data were in consistent with the literature reported (Hawas et al., 2012).

### 2.7. HCV protease inhibitory assay

Samples of 2 µl of culture broth fungi extracts dissolved in dimethyl sulfoxide (DMSO) were placed in each well of a Samples of 2 µl of culture broth fungi extracts dissolved in dimethyl sulfoxide (DMSO) were placed in each well of a 384-well microplate, then 8 µLof recHCV PR (0.5 µg/ml) were added, and the plate was briefly agitated. Finally, 10 µL of the freshly prepared substrate [Ac-Asp-Glu-Dap (QXLTM520)-Glu-Glu-Abu-COO-Ala-Ser-Cys(5-FAMsp)-$NH_2$] were added with sequential rotational shaking. The reaction mixture was incubated at 37 °C for 30 min. The fluorimetric analyses were performed on an automated TECAN GENios plate reader (Männedorf, Switzerland) with excitation and emission wavelengths at 485 and 520 nm, respectively. Each fungal extract was tested in triplicate. HCV PR inhibition (%) was calculated using the following equation:

$$\% \text{ Inhibition} = (F_{\text{substrate}} - F_{\text{test compound}}) \times 100/F_{\text{substrate}}$$

Where $F_{\text{substrate}}$ is the fluorescence of the enzyme and substrate only, and $F_{\text{test compound}}$ is the fluorescence of the assay mixture with the added compound (Love et al., 1996; Wei et al., 2009).

### 2.8. In vitro cytotoxic assay

Cytotoxicity of each fungus extract was tested against the human tumor cell lines of breast cancer (MCF-7) and liver cancer (Hep-G2) using MTT assay (Hussain et al., 1993). The cell lines were grown at 37 °C in 5% $CO_2$ and 95% humidity. A cell suspension containing 10000 cells was added to each well of the 96-well plate and left overnight. The cells were treated with fungal extracts under investigation, incubated for 24–72 h, and then the viability was assessed by. A standard concentration of 12.5 µg/ml of each culture broth extract was tested and was compared with doxorubicin (DOX) as drug reference.

### 2.9. Statistical analysis

The data of biochemical assessments are expressed as mean ± SD and the statistical analysis was carried out by using computer software Statistical Package for the Social Sciences (SPSS) version 16 (SPSS Inc. Released 2007, SPSS for Windows, and Version 16.0. Chicago, SPSS Inc.).

## 3. Results and discussion

Medicinal plants are well known to house numerous microorganisms within their tissues including fungi. Although Egypt has some of the most diverse and richest flora in the world and the medicinal plants

**Table 2**
Isolated fungi strains from Egyptian medicinal plants.

| Plant | Organ | Fungi species | Plant | Organ | Fungi species |
|---|---|---|---|---|---|
| *Plantago major* | Leaf | *Acremonium strictum* PML-1 | *Solanum nigrum* | Stem | *Aspergillus versicolor* SNS-11 |
| | | *Alternaria alternata* PML-2 | | | *Emercilla* sp. SNS-23 |
| | | *Bipolaris subpapendrofii* PML-12 | | | *Fusarium solani* SNS-31 |
| | | *Cochlibolus lunatus* PML-17 | | | *Pleospora tarda* SNS-47 |
| | | *Fusarium solani* PML-27 | | Root | *Fusarium solani* SNR-30 |
| | | *Pestalotia* sp. PML-46 | | | |
| | Root | *Fusarium solani* PMR-28 | | Leaf | *Nigrospora sphearica* SNL-42 |
| *Phoenix dactylifera* | Stem | *Alternaria alternate* PDL-5 | *Silybum marianum* | Leaf | *Fusarium oxysporum* SML-41 |
| | Root | *Fusarium* sp. PDR-34 | | Root | *Fusarium solani* SMR-29 |
| | | *Fusarium* sp. PDR-35 | | | *Trichoderma* sp. SMR-48 |
| | | *Fusarium solani* PDR-26 | | | |
| *Salvadora persica* | Stem | *Alternaria alternate* SPS-8 | *Euphorbia pulcherrima* | Stem | *Emercilla nidulans* EPS-20 |
| | | *Fusarium solani* SPS-32 | | | *Nigrospora sphaerica* EPS-38 |
| | | *Nigrospora sphaerica* SPS-40 | | | |
| | Leaf | *Alternaria alternate* SPL-7 | | Leaf | *Alternaria alternate* EPL-4 |
| | | *Emericella nidulans* SPL-25 | | | *Cochloobolus lunatus* EPL-18 |
| | | *Nigrospora sphaerica* SPL-39 | | | *Fusarium* sp. EPL-33 |
| | | | | | *Nigrospora sphaerica* EPL-37 |
| *Synadenium grantia* | Stem | *Chaetomium spirale* SGS-16 | *Punica granatum* | Leathery exocarp | *Alternaria alternata* PGL-3 |
| | | *Penicillium* sp. SGS-45 | | | |
| | Root | *Cunninghamella elegans* SGR-19 | | | *Penicillium* sp. PGL-44 |
| | | *Fusarium* sp. SGR-36 | | | |
| | Leaf | *Aspergillus sulphureus* SGL-9 | | | |
| | | *Chaetomium globosum* SGL-15 | | | |
| *Ruprechita saicifolia* | Stem | *Alternaria alternate* RSS-6 | *Ruprechita polystachya* | Stem | *Botryodiplodia theobromae* RPS-14 |
| | | *Aspergillus sydawii* RSS-10 | | | |
| | | *Emercilla nidulans* RSS-22 | | | |
| | Leaf | *Emericella nidulans* RSL-24 | | Leaf | *Botryodiplodia theobromae* RPL-13 |
| | | *Penicillium* sp. RSL-43 | | | *Emercilla nidulans* RPL-21 |

**Table 3**
Effect of secondary metabolites from the isolated endophytic fungi on HCV NS3/4A protease and cancer cell lines.

| Fungi species | HCV protease | | Breast cancer (MCF-7) | | Liver cancer (HEP-G2) | |
|---|---|---|---|---|---|---|
| | Inhibitory (%) 100 µg/ml | $IC_{50}$ (µg/ml) | Inhibitory (%) 100 µg/ml | $IC_{50}$ (µg/ml) | Inhibitory (%) 100 µg/ml | $IC_{50}$ (µg/ml) |
| *Acremonium strictum* PML-1 | 33.8 ± 5.43 | – | −8.0 ± 10.4 | – | 1.6 ± 1.3 | – |
| *Alternaria alternata* PGL-3 | 105.80 ± 0.17 | 17.0 ± 4.3 | 29.9 ± 5.3 | – | 33.7 ± 0.9 | – |
| *Aspergillus sulphureus* SGL-9 | 27.3 ± 3.9 | – | 8.8 ± 3.9 | – | 11.7 ± 4.3 | – |
| *Aspergillus versicolor* SNS-11 | 33.8 ± 5.8 | – | 8.8 ± 2.8 | – | 24.9 ± 2.2 | – |
| *Bipolaris subpapendrofii* PML-12 | 40.0 ± 3.4 | – | 2.8 ± 8.4 | – | 24.8 ± 8.2 | – |
| *Botryodiplodia theobromae* RPL-13 | 50.3.2 ± 4.4 | > 100 | 32.2 ± 4.4 | – | 36.7 ± 2.5 | – |
| *Chaetomium globosum* SGL-15 | 33.2 ± 8.8 | – | −28.3 ± 3.8 | – | −36.1 ± 8.6 | – |
| *Chaetomium spirale* SGS-16 | 36.3 ± 7.9 | – | 11.3 ± 7.9 | – | 5.4 ± 7.5 | – |
| *Cochlibolus lunatus* PML-17 | 103.7 ± 1.43 | 20.5 ± 2.5 | −34.6 ± 13.4 | – | −25.3 ± 5.5 | – |
| *Emercilla* sp. SNS-23 | 47.7 ± 5.6 | – | 37.7 ± 5.6 | – | 49.1 ± 3.1 | |
| *Emerecilla nidulans* RPL-21 | 70.0 ± 1.6 | 54.6 ± 2.2 | 95.9 ± 0.2 | 12.5 ± 0.5* | 96.5 ± 0.4 | 56.0 ± 5.5 |
| *Emericella nidulans* RSL-24 | – | – | 96.8 ± 0.4 | 10.8 ± 0.1* | 97.7 ± 0.3 | 20.3 ± 0.8* |
| *Emericella nidulans* RSS-22 | – | – | 94.0 ± 1.6 | 46.8 ± 3.5 | 96.4 ± 0.4 | 14.8 ± 2.2 |
| *Fusaium solani* SMR-29 | - | – | 98.8 ± 1.4 | 49.6 ± 7.5 | 98.3 ± 0.9 | 57.9 ± 9.4 |
| *Fusarium oxysporum* SML-41 | 12.7 ± 8.5 | – | 98.5 ± 0.1 | 11.0 ± 0.8* | 98.8 ± 0.2 | 24.0 ± 1.1* |
| *Fusarium solani* SNS-31 | 62.6 ± 2.7 | 82.2 ± 3.2 | 76.2 ± 3.0 | > 100 | 91.3 ± 0.8 | 65.8 ± .4.4 |
| *Fusarium* sp. SGR-36 | 37.2 ± 4.6 | – | −40.6 ± 4.6 | – | −24.2 ± 8.7 | – |
| *Fusarium* sp. PDR-35 | 46.8 ± 5.8 | – | −9.8 ± 11.8 | – | 6.4 ± 5.4 | – |
| *Fusarium* sp. PDR-34 | 31.0 ± 5.6 | – | −26.1 ± 5.6 | – | −23.9 ± 5.4 | – |
| *Nigrospora sphaerica* EPS-38 | 91.23 ± 1.9 | 33.6 ± 3.4 | 41.3 ± 8.5 | – | 70.7 ± 7.1 | |
| *Penicillium* sp. RSL-43 | 50.2 ± 2.8 | > 100 | 94.6 ± 1.8 | 13.7 ± 1.8* | 90.6 ± 6.1 | 37.9 ± 1.9 |
| *Penicillium* sp. SGS-45 | 33.5 ± 4.8 | – | −34.5 ± 4.8 | – | −45.1 ± 2.5 | – |
| *Penicillium* sp. PGL-44 | 33.1 ± 6.2 | – | 46.1 ± 6.2 | – | 53.2 ± 2.9 | > 100 |
| *Pleospora trada* SNS-47 | 22.6 ± 4.7 | – | −33.5 ± 1.7 | – | 1.5 ± 4.6 | – |
| *Trichoderma* sp. SMR-48 | 40.5 ± 6.1 | – | 98.5 ± 0.1 | 63.0 ± 2.3* | 98.7 ± 0.1 | 28.7 ± 1.5 |

* HCV protease inhibitor 2 activity was 0.5 ± 1.5 µM.
*The observations were averages of 3 replicates. The data of biochemical assessments are expressed as mean ± SD.

**Table 4**
18S rRNA Identification of bioactive *A. alternata* PGL-3 and *E. nidulans* RPL-21.

| Code | Fungus strain | Accession | Max score | Total score | Max. identity % |
|---|---|---|---|---|---|
| PGL-3 | *Alternaria alternata* | HM216191.1 | 257 | 257 | 89 |
| RPL-21 | *Emericella nidulans* | AB008403.1 | 2082 | 1127 | 99 |

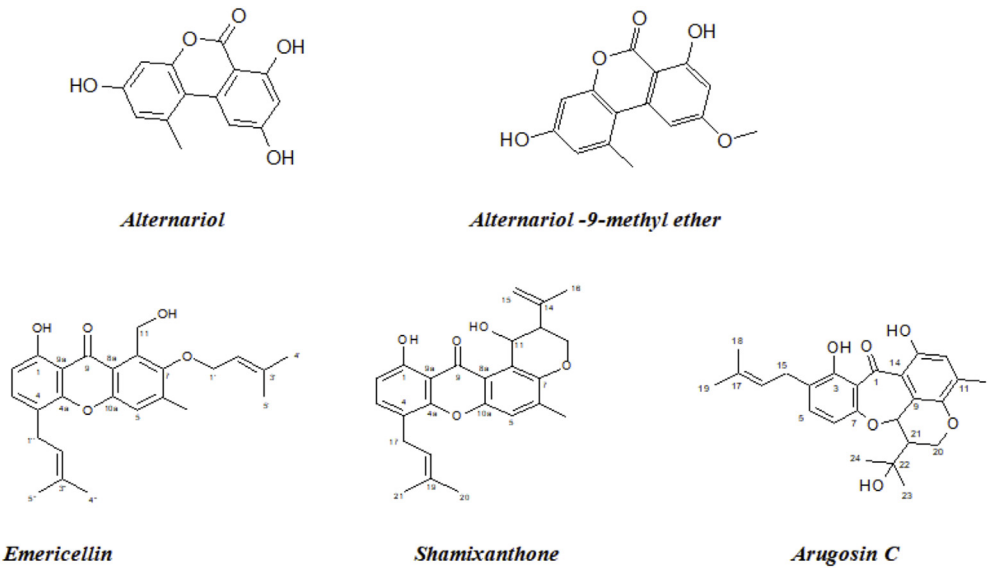


**Fig. 1.** Chemical structure of isolated compounds.

of Egyptian pharmacopoeia have been used in folk medicine since a long time, a few studies on their associated fungi are reported (Aly et al., 2008; El-Zayat et al., 2008; Selim et al., 2014). Thus, from the fresh tissues of 10 collected Egyptian plants, 48 endophytic fungi were isolated and their culture broth extracts were evaluated for anti-HCV protease and cytotoxic activities. This is the first report on the endophytic fungi isolated from the selected Egyptian medicinal plant compiled in Table 2.

### 3.1. HCV NS3/4A protease inhibitory action

The anti HCV protease activity was carried out for ethyl acetate extracts of the isolated endophytic fungal strains at 100 µg/ml concentration using the hepatitis virus C NS3 protease inhibitor 2 as a positive control. Among forty eight endophytic fungal strains, only 5 fungal extracts showed potential HCV protease inhibitory activity. The extracts of *A. alternata* PGL-3*, C. lunatus* PML-17 and *N. sphaerica* EPS-38 demonstrated the most potent inhibitory effect against HCV NS3/4A with $IC_{50}$ 17.0, 20.5 and 33.5 µg/ml, respectively (Table 3). On the other hand, *E. nidulans* RPL-21 and *F. solani* SNS-31 extracts showed mild inhibitory activity with $IC_{50}$ 54.6 and 82.2 µg/ml, respectively. These results suggested that the endophytic fungal extracts can be considered as a new source for active compounds against HCV which warrants for further investigation of their chemical constituents. Furthermore, this is the first reporting of active fungal endophytes, *A. alternata* PGL-3, *C. lunatus* PML-17 and *N. sphaerica* EPS-38 from the Egyptian medicinal plants *P. granatum, P. major* and *E. pulcherrima*, respectively, as promising source of HCV protease inhibitors.

### 3.2. Cytotoxic activity

*In vitro* cytotoxic activity of 48 isolated fungal extracts were evaluated against human breast cancer cell lines (MCF-7) and liver cancer cell lines (HEP-G2). As can be seen from the data in Table 3, the extracts of *E. nidulans* RSL-24, *E. nidulans* RPL-21, *F. oxysporum* SML-41 and *Penicillium* sp. RSL-43 exhibited potent cytotoxic activity against MCF-7 with $IC_{50}$ values of 10.8, 11.0, 12.5 and 13.7 µg/ml at a concentration of 12.5 µg/ml while the extracts of *E. nidulans* RSS-22 and *F. solani* SMR-29 at a concentration of 100 µg/ml revealed mild cytotoxic activity with $IC_{50}$ values of 46.8 and 49.6 µg/ml, respectively. Morover, at a concentration of 12.5 µg/ml, the extracts of *E. nidulans* RSL-24 and *F. oxysporum* SML-41 displayed potent cytotoxic activity against HEP-G2 with $IC_{50}$ values of 20.3 and 24.0 µg/ml, respectively. While at concentration of 100 µg/ml, the cytotoxic activity of *E. nidulans* RSS-22, *Trichoderma* sp. SMR-48 and *Penicillium* sp. RSL-43 was less pronounced with $IC_{50}$ values of 14.8, 28.7 and 37.9 µg/ml, respectively. These results are also first reporting of the active endophytes *E. nidulans* RSL-24, *E. nidulans* RPL-21, *F. oxysporum* SML-41, and *Penicillium* sp. RSL-43 from the host plant *R. saicifolia, R. polystachya, S. marianum* and *R. saicifolia*, respectively, as cytotoxic agents, which supported the hypothesis that the Egyptian medicinal plants are potential hosts of endophytes with medicinal properties.

*A. alternata* demonstrated a broad range of bioactivities (Soltani and Moghaddam, 2014). From the aforementioned results, *A. alternata* PGL-3 extract is the most effective HCV protease inhibitor. Interestingly, the extract of *A. alternate PGL-3* possessed a low cytotoxic effect on both liver cancer cell lines (HEP-G2) and breast cancer cell lines (MCF-7) even at a concentration about fivefold that of the $IC_{50}$ of antiviral (Table 3). A previous study reported relatively low acute toxicity of *Alternaria* toxins as compared to other mycotoxins (Pero et al., 1973). However, *E. nidulans* RPL-21 extract was unique to exhibit anti-HCV protease effect with very high cytotoxicity when compared with the extracts of the same fungus species isolated from other plants which enhance to follow up the phytochemical study in order to identify their constituents.

### 3.3. Secondary metabolites from active endophytic fungi

From the previous bioassays, the results showed that two endophytic fungi *A. alternata* PGL-3 and *E. nidulans* RPL-21 exhibited the highest anti-HCV protease and cytotoxic activities, respectively, which were subjected to 18S rDNA-identification (Table 4). The EtOAc extracts of these active endophytic cultures were chromatographed repeatedly by silica gel, Sephadex LH-20, thus two dibenzopyrone derivatives representing the major mycotoxins were isolated from *A. alternata* PGL-3 extract and identified as alternariol, and alternariol 9-methyl ether (Fig. 1). Despite the genotoxic, and cytotoxic properties of alternariol (AOH), and alternariol monomethyl ether, (Pfeiffer et al., 2007), the compounds were reported for a minor effect in the genotoxicity of *A. alternata* extracts (Schwarz et al., 2012). However, studies on their prospective biological activities still ongoing (Grover and Lawrence, 2017). Recently alternariol 9-methyl ether was isolated from *Alternaria* sp. from *Salvia miltiorrhiza* roots, exhibited antibacterial activity with $IC_{50}$ ranging from 6.00 to 38.27 µg/mL (Lou et al., 2016). Alternariol analogues were isolated from endolichenic fungi *Nigrospora* and *Phialophora* where alternariol and alternariol 9-methyl ether

showed antiviral activity against HSV, with $IC_{50}$ values of 13.5 and 21.3 μM, and Selectivity Index(SI) values of 26.5 and 17.1, respectively (He et al., 2012). On the other hand, two xanthones; emericellin, shamixanthone and one anthraquinone derivative augosin C (Fig. 1) were identified from the extract of *E. nidulans* RPL-21 which exhibited antiviral and cytotoxic activities. However, shamixanthone and emericellin showed either only marginal or no antitumor activity *in vitro* against human tumor cell lines (Kralj et al., 2006; Pornpakakul et al., 2006) while arugosin C was inactive against HCV NS3/4A (Hawas et al., 2012).

The study highlighted that endophytic fungi *A. alternate* isolated from *Punica granatum* plant displayed potential antiviral activity, however *A. alternata* from *P. major*, *S. persica* and *E. pulcherrima* were far from any observed effect. This is also, noticed for *E. nidulans* isolated from *R. polystachya* and those from *R. saicifolia, S. nigrum, S. persica* and *E. pulcherrima*, reflecting that both biomolecules and bioactivity of endophytes can be significantly influenced by the genus of host plant and the place in which the host plant grows (Hosseyni Moghaddam et al., 2013). These data are consistent with the recent studies which reported the antiviral activity of *Punica granatum* against Herpes simplex virus-2 and HCV (Arunkumar and Rajarajan, 2018; Rehman et al., 2018).

Since a virus mutates at a very high rate during replication, there is always a need for new antiviral molecules to overcome the anticipated evolution. Secondary metabolites from endophytes *A. alternata* may provide bioactive substances which might be modified for further therapeutic benefits.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101170.

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