

COMPARATIVE BIOLOGICAL EVALUATION OF SIX ENDOPHYTIC FUNGI ISOLATED FROM *VINCA ROSEA* LEAVES

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Abstract

In this study, a total of six endophytic fungi have been isolated from *Vinca rosea* (Apocynaceae) leaves growing in Egypt. The isolated fungi were identified morphologically and microscopically up to species to be; *Alternaria phragmospora*, *Aspergillus awamori*, *Penicillium duclauxii*, *Penicillium melinii*, *Nigrospora sphaerica* and *Mucor ramosissimus*. The extracts of the all identified fungi were screened biologically for antileukemic, cytotoxic, antimalarial, antileishmanial, antimicrobial, antioxidant and anti-inflammatory activities as well as for cannabinoid and opioid receptor binding affinities. Out of the six examined fungal extracts four exhibited promising antimalarial activities, four showed moderate antileukemic activities and two of them exhibited cytotoxic activities, three showed antioxidant activities, three exhibited anti-inflammatory activities, and the only one showed an antifungal activity. *Alternaria phragmospora* was the most active antimalarial agent inhibiting *Plasmodium falciparum* D6 and W2 clones with IC₅₀ values of 1.9 and 2.1 µg/mL, respectively. *Alternaria phragmospora* extract was subjected to liquid-liquid partition using 90% MeOH, hexane then BuOH and H₂O. MeOH and BuOH fractions exhibited strong activities against *Plasmodium falciparum* D6 clone with IC₅₀ values of 1.3 and 28 µg/mL and W2 clone with IC₅₀ values of 1.4 and 28 µg/mL, respectively. These results may be an excellent opportunity to get a new antimalarial drug derived from endophytic fungi.

Keywords: endophytic fungi, *Vinica rosea*, antileukemic, antimalarial, *Penicillium melinii*.

1. Introduction

Endophytic fungi are the fungi which live inside healthy plants either (intra or inter cellular) in a part or whole of their life cycle and don't cause apparent harmful effects (Petrini, 1991; Schulz et al., 1999). The word endophyte is originated from the Greek words, endon which means inside and phyton which means plant (Jalgaonwala et al., 2017).

Scientists indicated the ability of endophytic fungi to protect their host plants against a biotic stresses such as excess salt (Waller et al., 2005), drought (Elmi and West, 1995), and heat (Márquez et al., 2007). Furthermore, some endophytic fungi exhibited the power to protect their host plants from some biotic stresses such as infection by raising plant's microbial resistance through induction of phytoalexins production in addition to fungal ability to secrete antimicrobial metabolites inside the plant (Gao et al., 2010). Endophytic fungi are untapped reservoir for bioactive chemically novel metabolites with various chemical classes (Tenguria et al., 2011).

Over last years, A huge amounts of bioactive secondary metabolites have been isolated from endophytic fungi belonging to different chemical groups such as; tetralones (Kokubun et al., 2003), cytochalasins (Wagenaar et al., 2000), xanthonones (Krohn et al., 2009) terpenoids (Bilal et al., 2018), alkaloids (Metwaly et al., 2015), steroids (Hussain et al., 2009), polyketones (Zheng et al., 2018), quinones (Stierle and Stierle, 2015), phenolics (Das et al., 2018), isocoumarines (Orfali et al., 2015), isochromenes, (Metwaly, A.M. et al., 2014b), benzopyranones (Metwaly, A. et al., 2014), furandiones (Li et al., 2015), α -pyrones (Metwaly, A.M. et al., 2014a; Metwaly et al., 2017), and butenolide derivatives (Guo et al., 2016).

2. Materials and Methods

2.1. Fungal material

2.1.1. Isolation of endophytic fungi

The fungi were isolated from surface sterilized fresh leaves of an apparently healthy *Vinca rosea* (Apocynaceae) collected from Botanical Garden of Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. The leaves were rinsed with water and followed by surface sterilization in 70% EtOH for 1 min, rinsed again with sterilized water, then cut into small pieces (2 cm in length and width) and deposited in a petri dish containing PDA medium (200 g potato, 20 g glucose, and 15 g agar in 1 L distilled water, supplemented with 100 mg/ L chloramphenicol) and cultivated at 28°C for 3 days. The hypha tips were observed and transferred to new PDA plates and subcultured until pure culture was obtained.

2.1.2. Identification of endophytic fungi

The fungus was identified by the regional center for mycology and biotechnology, Cairo, Egypt. Pure colony of each fungal isolate was inoculated on different medium (potato-dextrose agar, Czapek's agar, Czapek's yeast extract agar (CYA) and malt extract agar) and incubated for 1-7 days. Cultural features for each fungal isolate in addition to microscopic examination of reproductive structure were recorded. identification was based on current universal keys (Fisher and Cook, 1998; Hoog et al., 2000) and on the data base identification program of the Regional Center for Mycology and Biotechnology (RCMB) for fungi using an Image Analysis System.

2.1.3. Mass cultivation

Each endophytic fungus was grown on PDA at 28 °C for 5 days. Ten pieces (0.5×0.5 cm²) of mycelial agar plugs were inoculated into ten 1000 mL Erlenmeyer

flasks containing sterilized 100 g Asian rice and 100 ml distilled water. The flasks were incubated under static conditions at room temperature for 40 days.

2.3. Extraction and fractionation

2.3.1. Preparation of total extracts

Each fungus was extracted by adding 1 L EtOAc (twice) to each flask and homogenized. The homogenized suspensions were collected, filtrated, concentrated under vacuum and prepared for biological assay.

2.3.2. Fractionation of *Alternaria phragmospora* extract

The ethyl acetate portion was evaporated to dryness and partitioned with hexane and 90% MeOH to afford the hexane fraction and MeOH fraction. The water portion was fractionated against *n*-butanol to afford the water fraction and the butanol fraction.

2.4. Antimalarial assay

Crude fungal extracts were tested for activity against chloroquine-sensitive (D6, Sierraleon) and -resistant (W2, Indo-china) strains of *Plasmodium falciparum* using previously reported method (Bharate et al., 2007). The anti-malarial screen tests samples for their ability to inhibit the chloroquine-sensitive (D6) and/or chloroquine-resistant (W2) *Plasmodium falciparum* protozoan. Crude extracts are initially tested against the D6 *P. falciparum* strain.

Primary Screen;

at 15867 ng/mL in duplicate, and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing $\geq 50\%$ inhibition proceed to the Secondary Assay.

Secondary Assay;

In the secondary antimalarial assay, samples dissolved to 20mg/mL (crude extracts and some column fractions) are tested at 47600, 15867, and 5289ng/mL and IC₅₀s (test concentration in ng/mL that affords 50% inhibition of the protozoan relative to negative and positive controls) vs. both the D6 and W2 strains are reported. Samples dissolved to 2mg/mL are tested at 4760, 1587, and 529ng/mL and IC₅₀s vs. both the D6 and W2 strains are reported. In addition to the *P. falciparum* strains, samples are tested in the VERO mammalian cell line as an indicator of general cytotoxicity. The selectivity indices (SI) – the ratio of VERO IC₅₀ to D6 or W2 IC₅₀ - are calculated. All IC₅₀s are calculated using the XLFit fit curve fitting software. The antimalarial drug controls chloroquine and artemisinin are used as positive controls

2.5. Antileishmanial assay

The anti-leishmanial screen (LEM) tests samples for their ability to inhibit *Leishmania donovani*, a fly-borne protozoan that causes visceral leishmaniasis. Crude extracts are initially tested in a Primary Screen.

Primary Screen;

at 80 μ g/mL in duplicate and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing $\geq 50\%$ inhibition proceed to the secondary assay using the previously reported method (Abdel-Mageed et al., 2012).

2.6. Antimicrobial screen

Crude extracts tested for antimicrobial activity against 90906, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Staphylococcus aureus* ATCC 2921, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Mycobacterium intracellulare* ATCC 23068 *Candida albicans* ATCC 90028, *Candida glabrata* ATCC90030, *Candida krusei* ATCC 6258, *Aspergillus fumigates* ATCC and *Cryptococcus neoformans* ATCC 90113, Ciprofloxacin and Amphotericin B were used as positive standards.

Primary Screen;

Crude extracts are initially tested at 50 μ g/mL in duplicate and percent inhibitions (% inh.) are calculated relative to negative and positive controls. Extracts showing $\geq 50\%$ inhibition proceed to the Secondary Assay.

Secondary Assay;

In the Secondary Assay, samples dissolved to 20mg/mL (crude extracts and some column fractions) are tested at 200, 40, 8 μ g/mL and IC₅₀s vs. all 10 microbial strains are reported. Samples dissolved to 2mg/mL (pure compounds and some column fractions) are tested at 20, 4, 0.8 μ g/mL and IC₅₀s vs. all 10 microbial strains are reported. Pure compounds that have an IC₅₀ of $\leq 7\mu$ g/mL in the Secondary OI assay proceed to the Tertiary Assay.

The MIC is the lowest test concentration (in μ g/mL) that inhibits the organism 100%. The MFC or MBC is the lowest test concentration (in μ g/mL) that kills the organism. While a pure compound may have an MIC, the cells may still be alive, just not growing. The MFC and MBC is a way to monitor the "cidality" or the killing ability of the test sample. All IC₅₀s are calculated using the XLFit fit curve fitting software.

2.7. Antileukemic assay

Human acute leukemia HL60 cells and human chronic leukemia K562 cells were purchased from American Type Culture Collection, Rockville MD, USA. Both cell lines were grown in suspension culture at 37 °C in RPMI-1640 medium supplemented with 10% non-dialysed fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL of penicillin and 10 μ g/mL of streptomycin. For the cell growth inhibition assay, HL60 and K562 cells were seeded at 1 x 10⁵ cells/well in Costar 24-well plates. Cells were allowed to grow undisturbed for 24 h before addition of test samples. After 48 h incubation with samples at 37 °C, viable cell counts were made by using the trypan blue exclusion method to assess cell viability (Roper and Drewinko, 1976).

2.8. Cytotoxicity Assay

Cytotoxicity was determined against HepG2 (human hepatocarcinoma), Vero (African green monkey kidney fibroblasts) and LLC-PK1 (pig kidney epithelial) cells as described earlier (Mustafa et al., 2004). Doxorubicin was used as a positive control.

2.9. Anti-inflammatory assays

Anti-inflammatory activity was determined in terms of the inhibition of NF- κ B-mediated transcription and inhibition of intracellular generation of reactive oxygen species (ROS) and nitric oxide (NO). Inhibition of NF- κ B mediated transcription was determined in human chondrosarcoma (SW1353) cells by a reporter gene assay as described earlier (Ma et al., 2007). Sp-1 was used as a control transcription factor to evaluate the toxicity of tested compounds in the same assay. Parthenolide was used as the positive control. Inhibition of intracellular NO production as a result of iNOS activity was assayed in mouse macrophages (RAW 264.7 cells) as described before

(Quang et al., 2006). Parthenolide was included in each assay as the positive control. Inhibition of intracellular ROS generation (antioxidant activity) was assayed in human promyelocytic leukemia (HL-60) cells by using DCFH-DA as described previously (Reddy et al., 2007). Trolox was used as a positive control.

2.10. Antioxidant assay

Antioxidant activity was determined by the DCFH-DA (20,70-dichlorofluorescein diacetate) method in myelomonocytic HL-60 cells as described earlier (Abbas et al., 2007). Briefly, for the assay, cells were plated at a density of 1.25×10^5 cells per well in 96-well plates. After treatment with different concentrations of the test samples for 30 min, cells were stimulated with 100 ng mL⁻¹ phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min. DCFH-DA (molecular probes, 5 mg mL⁻¹) was added and further Antimicrobial assay incubated for 15 min. Plates were read on a polar star at an excitation wavelength of 485 nm and emission at 530 nm to measure the level of DCF production.

2.11. Opioid and Cannabinoid Receptor Binding Assay

This assay is designed to use a series of controls to determine the binding affinity of the test extracts against Kappa (κ), Delta (δ) and Mu (μ) opioid receptors in addition to Cannabinoid Receptors CB 1 and CB 2. 10 μ M of a positive control [*nor*-Binaltorphimine dihydrochloride (κ), DPDPE (δ), or DAMGO (μ)] was used to ascertain non-specific binding (NSB) and 1% ethanol or DMSO in Tris-EDTA buffer was used to ascertain total binding. For Cannabinoid Receptor Binding Assay 10 μ M of a CP-55,940 was used to ascertain non-specific binding and 1% ethanol or DMSO in Tris-EDTA buffer was used to ascertain total binding. To eliminate the possibility of contamination in the test extracts, controls or the radioligand, wells with 1% ethanol or DMSO with no membrane were tested. The test have been done using a 96-well format as described in the scientific literature (Bradford, 1976; Kumarihamy et al., 2015).

3. Results and Discussions

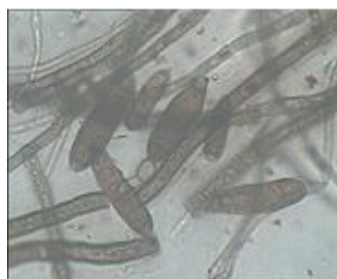
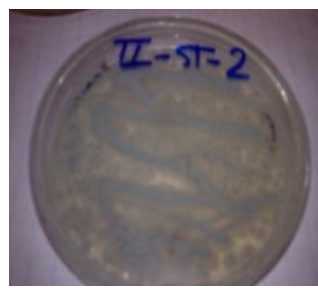
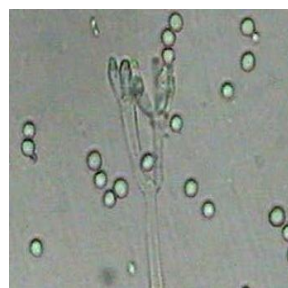
3.1. Isolation and identification of the endophytic fungi

In this study, a total of six endophytic fungal isolates were isolated on PDA from 50 leaf segments of *Vinca rosea* (Apocynaceae) growing in Egypt and identified as *Alternaria phragmospora*, *Aspergillus awamori*, *Penicillium duclauxii*, *Penicillium melinii*, *Nigrospora sphaerica* and *Mucor ramosissimus* (Figure 1). Morphological and microscopical characters of the identified fungi have been summarized in Table 1.

The fungal identification was carried out by the Regional Center for Mycology and Biotechnology, (Cairo, Egypt). The fungi were grown on Asian rice as a solid media and then extracted with ethyl acetate. All fungal extracts were biologically screened for antileukemic, cytotoxic, antimalarial, antileishmanial, antimicrobial, antioxidant and anti-inflammatory activities as well as for cannabinoid and opioid receptor binding affinity.

Table 1. Morphological and microscopical characters of the identified fungi

| | Culture Examination | Microscopic Examination |
|--------------------------------|---|---|
| <i>Alternaria phragmospora</i> | Colonies on PDA grayish brown became black in colour | Conidiophore; 3.0 μ m. Conidia; Cylindrical, brown, 2-9 longitudinal septa. 45X 9.5 μ m. Chlamydo spores; Abundant |
| <i>Aspergillus awamori</i> | Colonies fast-growing on malt media, usually consist of a compact white or yellow, mycelium with greenish black conidial heads. Reverse is colourless | Conidiophores; Thick up to 20.0 μ m. Conidia globose, 3.5 in diam. Chlamydo spore was absent. Sterigmata in two series. Conidial heads; mostly 100-500 μ m. Vesicles; globose, 30.9 μ m |
| <i>Penicillium duclauxii</i> | Colonies on CYA at 25°C gives mycelium white to pale yellow; heavy dull green spores with yellow reverse. | Penicillus type; Terverticillate and may biverticillate at certain isolates. Rami; 13.2X 3.5 μ m. Metulae; 10.5X3.0 μ m. Phialides; 9.6X2.0 μ m. Conidia subglobose, 3.6 μ m. |
| <i>Penicillium melinii</i> | Colonies on CYA at 25°C gives mycelium white or less commonly pale yellow; heavy dull green spores with orange to brown reverse. | Penicillus type; Biverticillate. Metulae; 10.7X3.6 μ m. Phialides; 7.3X2.8 μ m. Conidia subglobose, 3.5 μ m. |
| <i>Nigrospora sphaerica</i> | Colonies on PDA first white, later brown to black due to abundant sporulation. | Conidiophores; 9.0 μ m. Conidia spherical or oblate opaque, 16.0 μ m |
| <i>Mucor ramosissimus</i> | Colonies on PDA grayish at 28°C, gives mycelium white | Conidiophores; 18.0 μ m. Sporangia; Blackish, spherical, 75 μ m. Sporangiospores; Ellipsoidal, 6.2X5.0 μ m. |

*Alternaria phragmospora**Aspergillus awamori**Penicillium duclauxii**Penicillium melinii**Nigrospora sphaerica**Mucor ramosissimus***Fig.1.** The isolated fungi under a microscope and as pure strains on malt agar plate

3.2. Antimalarial assay results

The extract of *Alternaria phragmospora*, *Nigrospora sphaerica*, *Aspergillus awamori* and *Penicillium melinii* showed promising antimalarial activities against *Plasmodium falciparum* D6 clone with IC₅₀ values of 1.9, 9.7, 11 and 19 µg/mL and against W2 clone with IC₅₀ values of 2.1, 9.7, 18 and 15 µg/mL, respectively as shown in Table 2 and figure 2. The most active endophytic fungal extract (*Alternaria phragmospora*) was subjected to liquid-liquid partition using 90% MeOH, hexane then BuOH and H₂O to give MeOH, BuOH, hexane and H₂O fractions. Each fraction was examined for antimalarial activity. Water and hexane fractions were found to be inactive, but MeOH and BuOH fractions showed antimalarial activities against *Plasmodium falciparum* D6 clone with IC₅₀ values of 1.3 and 28 µg/mL, respectively, and W2 clone with IC₅₀ values of 1.4 and 28 µg/mL, respectively.

Table 2: Antimalarial assay results

| | <i>P. falciparum</i> D6 IC ₅₀ (µg/ml) | <i>P. falciparum</i> W2 IC ₅₀ (µg/ml) |
|--|---|---|
| <i>Alternaria phragmospora</i> | 1.9 | 2.1 |
| <i>Alternaria phragmospora</i> BuOH fraction | 28 | 28 |
| <i>Alternaria phragmospora</i> MeOH fraction | 1.3 | 1.4 |
| <i>Aspergillus awamori</i> | 11 | 18 |
| <i>Mucor ramosissimus</i> | NA ^a | NA ^a |
| <i>Nigrospora sphaerica</i> | 9.7 | 9.7 |
| <i>Penicillium duclauxii</i> | NA ^a | NA ^a |
| <i>Penicillium melinii</i> | 19 | 15 |
| Artemisinin | 0.026 | 0.026 |

^a NA: Extracts having IC₅₀ value > 25.8 µg/ml

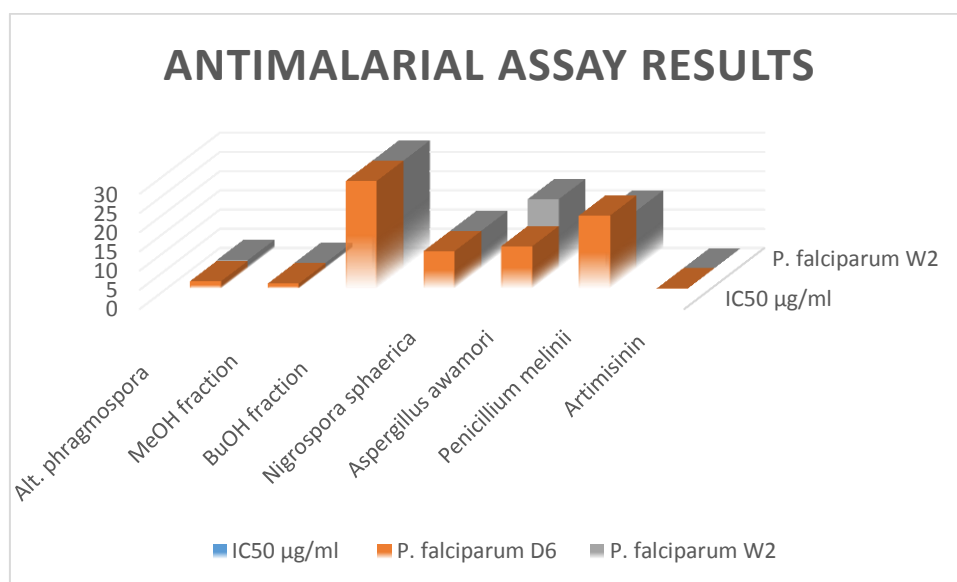


Fig. 2. Antimalarial assay results

3.3. Antileishmanial assay results

All fungal extracts of *Penicillium duclauxii*, *Nigrospora sphaerica*, *Alternaria phragmospora*, *Aspergillus awamori*, *Mucor ramosissimus*, *Penicillium melinii* inhibited the growth of *L. donovani* growth with % Inhibition of 47, 12.5, 11.9, 10.8, 8.4 and 7.4, respectively.

Table 3: Antileishmanial assay results

| | <i>L. donovani</i> | |
|--------------------------------|--------------------|--------------------------|
| | % Inh. | IC ₅₀ (µg/ml) |
| <i>Alternaria phragmospora</i> | 11.9 | NA ^a |
| <i>Aspergillus awamori</i> | 10.8 | NA ^a |
| <i>Mucor ramosissimus</i> | 8.4 | NA ^a |
| <i>Nigrospora sphaerica</i> | 12.5 | NA ^a |
| <i>Penicillium duclauxii</i> | 47 | NA ^a |
| <i>Penicillium melinii</i> | 7.4 | NA ^a |

^aNA: Extracts having IC₅₀ value > 80µg/ml

3.4. Antimicrobial assay results

All endophytic fungal extracts have been examined for their ability to inhibit the growth of a panel of 5 bacteria and 5 fungi those are pathogenic to humans including; *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium intracellulare*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Aspergillus fumigates* and *Cryptococcus neoformans*. Some extracts exhibited inhibitory effects with different percentages against the examined organisms ranging from 1 to 37 (Table 4 and 5), while the endophytic fungus *Nigrospora sphaerica* showed antifungal activity against *Aspergillus fumigatus* with an IC₅₀ value of 49 µg/ml

Table 4: Antibacterial assay results

| | <i>S. aureus</i> | MRSA | <i>E. coli</i> | <i>P. aeruginosa</i> | <i>M. Intracellulare</i> |
|--------------------------------|------------------|--------|----------------|----------------------|--------------------------|
| | % Inh. | % Inh. | % Inh. | % Inh. | % Inh. |
| <i>Alternaria phragmospora</i> | 3 | 2 | - | - | - |
| <i>Aspergillus awamori</i> | - | 7 | 24 | 1 | - |
| <i>Mucor ramosissimus</i> | 1 | 2 | - | - | - |
| <i>Nigrospora sphaerica</i> | 1 | 2 | 22 | - | - |
| <i>Penicillium duclauxii</i> | - | - | 10 | 3 | 1 |
| <i>Penicillium melinii</i> | - | 11 | 21 | - | - |

^aNA: Extracts having IC₅₀ value > 50µg/ml

Table 5: Antifungal assay results

| | <i>C. albicans</i> | | <i>C. glabrata</i> | | <i>C. krusei</i> | | <i>A. fumigatus</i> | | <i>C. neoformans</i> | |
|--------------------------------|--------------------|------------------|--------------------|------------------|------------------|------------------|---------------------|------------------|----------------------|------------------|
| | % Inh. | IC ₅₀ | % Inh. | IC ₅₀ | % Inh. | IC ₅₀ | % Inh. | IC ₅₀ | % Inh. | IC ₅₀ |
| <i>Alternaria phragmospora</i> | 18 | NA ^a | 7 | NA ^a | 41 | - | - | NA _a | 37 | NA ^a |
| <i>Aspergillus awamori</i> | 2 | NA ^a | 2 | NA ^a | 27 | - | 6 | NA _a | 9 | NA ^a |
| <i>Mucor ramosissimus</i> | 7 | NA ^a | 5 | NA ^a | 16 | - | 2 | NA _a | 3 | NA ^a |
| <i>Nigrospora sphaerica</i> | 5 | NA ^a | 3 | NA ^a | 17 | - | 61 | 49 | 7 | NA ^a |
| <i>Penicillium duclauxii</i> | | NA ^a | 1 | NA ^a | 25 | - | - | NA _a | | NA ^a |
| <i>Penicillium melinii</i> | 2 | NA ^a | 0 | NA ^a | 6 | - | 9 | NA _a | 5 | NA ^a |

^aNA: Extracts having IC₅₀ value > 50µg/ml

3.5. Antileukemic and cytotoxic assay results

Alternaria phragmospora, *Penicillium melinii*, *Penicillium duclauxii*, *Aspergillus awamori* extracts showed moderate antileukemic activities against K562 cells with IC₅₀ values of 0.035, 0.056, 0.05 and 0.07 µg/ml and against HL60 cells with IC₅₀ values of 0.045, 0.23, 0.031 and 0.04 µg/ml, respectively. These fungi have been chosen for cytotoxic activity examination against HepG2 (human hepatocarcinoma), Vero (African green monkey kidney fibroblasts) and LLC-PK1 (pig kidney epithelial). The endophytic fungal extracts of *Alternaria phragmospora* and *Aspergillus awamori* showed moderate cytotoxic activity against LLC-PK₁₁ with IC₅₀ values of 85 and 67 µg/ml, respectively. In addition, *Alternaria phragmospora* showed activity against HepG2 with an IC₅₀ value of 55 µg/ml as shown in Table 6.

Table 6: Antileukemic and cytotoxic assay results

| | IC ₅₀ (µg/ml) | | | | |
|--------------------------------|--------------------------|---------------------|-----------------|-----------------|-----------------|
| | Leukemia K562 cells | Leukemia HL60 cells | HepG2 | Vero | LLC-PK1 |
| <i>Alternaria phragmospora</i> | 0.035 | 0.045 | 55 | NA ^a | 85 |
| <i>Aspergillus awamori</i> | 0.07 | 0.04 | NA ^a | NA ^a | 67 |
| <i>Mucor ramosissimus</i> | NA ^a | NA ^a | NA ^a | NA ^a | NA ^a |
| <i>Nigrospora sphaerica</i> | NA ^a | NA ^a | NA ^a | NA ^a | NA ^a |
| <i>Penicillium duclauxii</i> | 0.05 | 0.031 | NA ^a | NA ^a | NA ^a |
| <i>Penicillium melinii</i> | 0.056 | 0.23 | NA ^a | NA ^a | NA ^a |

^aNA: Extracts having IC₅₀ value > 100µg/ml

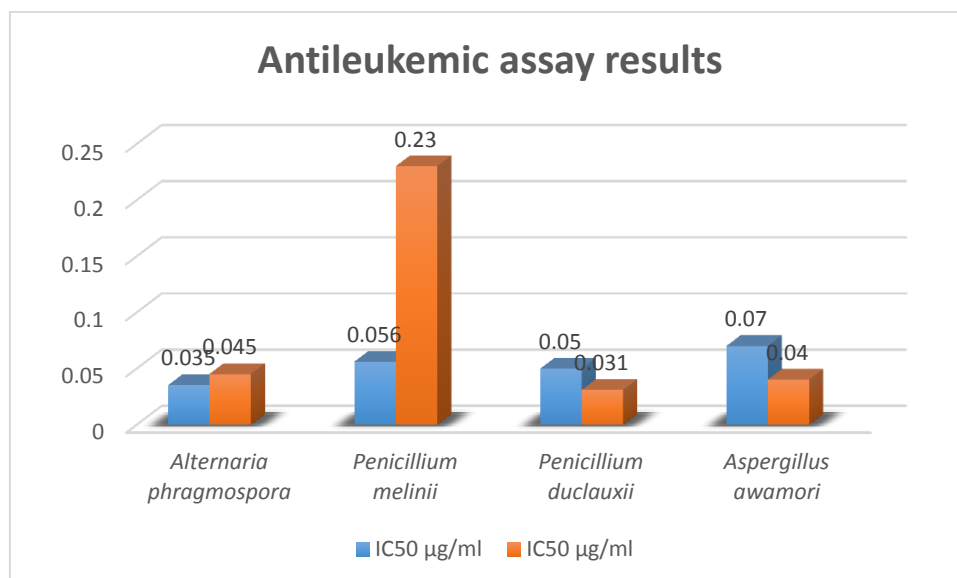


Fig.3. Antileukemic assay results

3.6. Anti-inflammatory assay results

All the fungal extracts have been subjected to screen anti-inflammatory activities. The activity was determined in terms of the inhibition of nuclear factor (NF- κ B) mediated transcription and inhibition of intracellular generation of reactive oxygen species (ROS) and nitric oxide synthase (NOS). The endophytic fungal extracts of *Alternaria phragmospora*, *Penicillium melinii* and *Mucor ramosissimus* showed nitric oxide synthase inhibitory (iNOS) activities with IC₅₀ values of 37, 64 and 61 μ g/ml, respectively, as shown in Table 7.

Table 7: Anti-inflammatory assay results

| | Anti-inflammatory activity assay results in IC ₅₀ (μ g/ml) | | |
|--------------------------------|--|--------------------------------|-----------------|
| | <i>iNOS</i> | <i>NF-κB</i> | <i>SP-1</i> |
| <i>Alternaria phragmospora</i> | 37 | NA ^a | NA ^a |
| <i>Aspergillus awamori</i> | NA ^a | NA ^a | NA ^a |
| <i>Mucor ramosissimus</i> | 61 | NA ^a | NA ^a |
| <i>Nigrospora sphaerica</i> | NA ^a | NA ^a | NA ^a |
| <i>Penicillium duclauxii</i> | NA ^a | NA ^a | NA ^a |
| <i>Penicillium melinii</i> | 64 | NA ^a | NA ^a |

^a NA: Extracts having IC₅₀ value > 100 μ g/ml

3.7. Anti-oxidant assay results

Anti-oxidant activities were determined by the DCFH-DA (20,70-dichlorofluorescein diacetate) method in myelomonocytic HL-60 cells. The endophytic fungal extracts of *Aspergillus awamori*, *Nigrospora sphaerica* and *Mucor ramosissimus* showed anti-oxidant activities with IC₅₀ values of 22, 18 and 11 μ g/ml, respectively.

Table 8: Antioxidant assay results

| | Antioxidant activity (IC ₅₀ µg/ml) |
|--------------------------------|---|
| <i>Alternaria phragmospora</i> | NA ^a |
| <i>Aspergillus awamori</i> | 22 |
| <i>Mucor ramosissimus</i> | 11 |
| <i>Nigrospora sphaerica</i> | 18 |
| <i>Penicillium duclauxii</i> | NA ^a |
| <i>Penicillium melinii</i> | NA ^a |

^aNA: Extracts having IC₅₀ value > 100µg/ml

3.8. Opioid receptors binding assay results

The extracts of *Aspergillus awamori*, *Penicillium melinii* and *Penicillium duclauxii* exhibited very weak opioid receptors binding affinities against Delta receptors with % inhibition values of 2.9, 2.2 and 4.2 and against Mu receptors with % inhibition values of 5.7, 2.1 and 0.8, respectively. *Nigrospora sphaerica* extract weakly inhibited Delta receptors with a value of 15.2%. All the examined fungal extracts didn't show any activity against Kappa receptors.

Table 9: Opioid receptors binding assay results

| | Kappa receptors %Inh. | Delta receptors % Inh. | Mu receptors % Inh. |
|--------------------------------|--------------------------|---------------------------|------------------------|
| <i>Alternaria phragmospora</i> | - | - | - |
| <i>Aspergillus awamori</i> | - | 2.91 | 5.70 |
| <i>Mucor ramosissimus</i> | - | - | - |
| <i>Nigrospora sphaerica</i> | - | 15.16 | - |
| <i>Penicillium duclauxii</i> | - | 4.21 | 0.80 |
| <i>Penicillium melinii</i> | - | 2.24 | 2.13 |

3.9. Cannabinoid receptors binding assay results

Aspergillus awamori, *Nigrospora sphaerica* and *Mucor ramosissimus* extracts were found to have weak inhibitory effects against CB 1 receptors with % inhibition of 8.7, 16.6 and 6 and against CB 2 receptors with % inhibition of 8.6, 8.9 and 1.5, respectively. In addition, *Penicillium melinii* extract showed weak inhibitory effects against CB 1 receptors with % inhibition of 20.2, while *Penicillium duclauxii* inhibited CB 2 receptors with % inhibition of 0.2.

Table 10: Cannabinoid receptors binding assay results

| | CB 1 receptors (%Inh) | CB 2 receptors (%Inh) |
|--------------------------------|-----------------------|-----------------------|
| <i>Alternaria phragmospora</i> | - | - |
| <i>Aspergillus awamori</i> | 8.72 | 8.58 |
| <i>Mucor ramosissimus</i> | 6.02 | 1.52 |
| <i>Nigrospora sphaerica</i> | 16.65 | 8.87 |
| <i>Penicillium duclauxii</i> | - | 0.15 |
| <i>Penicillium melinii</i> | 20.18 | - |

Conclusion

Six endophytic fungi have been isolated from *Vinca rosea* (Apocynaceae) leaves growing in Egypt. The isolated fungi were identified morphologically and microscopically up to species level. The fungal extracts were screened biologically for antileukemic, cytotoxic, antimalarial, antileishmanial, antimicrobial, antioxidant and anti-inflammatory activities as well as for cannabinoid and opioid receptor binding affinities. Several fungal extracts exhibited promising biological activities. The most promising activity was for the extract of *Alternaria phragmospora* as an antimalarial agent. *Alternaria phragmospora* extract inhibited *Plasmodium falciparum* D6 and W2 clones with IC₅₀ values of 1.9 and 2.1 µg/mL, respectively. *Alternaria phragmospora* extract was subjected to liquid-liquid partition using 90% MeOH, hexane then BuOH and H₂O. MeOH and BuOH fractions exhibited strong activities against *Plasmodium falciparum* D6 clone with IC₅₀ values of 1.3 and 28 µg/mL and W2 clone with IC₅₀ values of 1.4 and 28 µg/mL, respectively. The fungus *Alternaria phragmospora* may open a new hope and promising chance to get a new antimalarial drug derived from endophytic fungi.

Acknowledgments

We are grateful to the Egyptian Government and National Center for Natural Products Research, School of Pharmacy, University of Mississippi, for financial support. We are also thankful to Drs. Melissa Jacob and Babu Tekwani for antimicrobial and antileishmanial assays. This investigation was conducted in part in a facility constructed with support from the research facilities improvement program C06 RR-14503-01 from the NIH NCCR. This work is supported in part by United States Department of Agriculture ARS Specific Cooperative Agreement No. 58-6408-2-0009.

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التقييم البيولوجي المقارن لستة فطريات معزولة من أوراق نبات الوينكا

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الخلاصة : في هذه الدراسة ، تم عزل ما مجموعه ستة الفطريات الفطرية من أوراق نبات الوينكا التي تنمو في مصر. تم التعرف على الفطريات المعزولة شكلياً ومجهرياً حتى تكون الأنواع ؛
Penicillium و Penicillium duclauxii و Aspergillus awamori و Alternaria phragmospora
و Nigrospora sphaerica و Mucor ramosissimus و melinii
تم فحص خلاصات من الفطريات التي تم تحديدها جميعها بيولوجياً بحثاً عن تأثير مضاد لسرطان الدم وتأثير
مضاد للخلايا السرطانية وتأثير مضاد للشمانيا وتأثير مضاد للملاريا وتأثير مضاد للميكروبات و تأثير مضادات
الأكسدة وتأثير مضاد للالتهابات