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Cytotoxicity and antimicrobial activity of alkaloids extracted from *Catharanthus roseus* associated endophytic fungi

Eman Abdullah Mohammad Ali[1,*], Tahany Mohammad Ali Abdel-Rahman[1] and Hedia Allah Ebrahim

[1]Department of Botany and Microbiology, Faculty of Science, Cairo University, Giza, Egypt.
Email: emanali@sci.cu.edu.eg

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ABSTRACT

Aims: The aim of this study was to isolate the endophytic fungi from *C. roseus* and screen the cytotoxicity and antimicrobial activity of alkaloids extracted from these fungi.
Methodology and results: A total of 56 endophytic fungal isolates were screened from *C. roseus* plant parts. *A. fumigatus* and *F. oxysporum* were the most frequent species. Determinations of alkaloids extracted from the most dominant endophytic fungal species were done. The highest significant total alkaloids productions were by *A. fumigatus* and *A. niger*, while the least significant one was by *Botrytis cinera*. Antimicrobial assay of endophytic fungal extracts indicated that both *A. niger* and *F. oxysporum* exhibited significant antimicrobial activities, while *A. fumigatus* exerted the least activity. In vitro cytotoxicity assay of the endophytic fungal extracts was done against human breast cancer (MCF-7) and liver cancer (HEPG-2) cell lines using SRB assay method. *A. niger* extract showed potential cytotoxic effect on MCF-7 cell line with IC50 value of 42.1 µg/mL, while the least cytotoxic effect was exhibited by *F. oxysporum* on MCF-7 cell line with IC50 value of 66.9 µg/mL. Gas Chromatography Mass Spectroscopy (GC-MS) was used for analysis of alkaloids in mycelial and filtrate extracts of *A. niger*, where eleven compounds were detected.
Conclusion, significance and impact of study: Alkaloids extracted from *Catharanthus roseus* associated endophytic fungi had cytotoxic and antimicrobial activities.

Keywords: Endophytic fungi, *Catharanthus roseus*, alkaloids, antimicrobial activity, cytotoxicity

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INTRODUCTION

The original source for both ancient and modern medicine is mostly the medicinal plant (Karimi and Jaafar, 2011). More than 70% of the antimicrobial and 60% of the anticancer drugs in medicinal uses are natural products or their derivatives (McAlpine *et al.*, 2005).

Alkaloids are heterogeneous group of 2500 basic nitrogen containing compounds, found in about 15% of all vascular land plants constituting more than 150 plant families, widely distributed in higher plants particularly the dicotyledons (Mohan *et al.*, 2012).

Catharanthus roseus (Madagascar Periwinkle) is an important medicinal plant belong to family Apocyanacae. It is a main source for alkaloids which have anticancer, antimicrobial and antidiabetic activities (Jaleel *et al.*, 2006; Gajalakshmi *et al.*, 2013). Vincristine and vinblastine are the most common terpenoid indole alkaloids produced by *C. roseus* and are used as anticancer (Balaabirami and Patharajan, 2012; Kumar *et al.*, 2015; Yamamoto *et al.*, 2016) and anti-microbial agents (Grellier *et al.*, 1999). Vincamine and vinpocetine are used for treatment of cerebrovascular and cognitive disorders, and to reduce the effects of stroke types (Chen *et al.*, 1995; Chevallier, 1996). Ajmalicine and serpentine are monoterpenic indole alkaloids which are used as anti-hypertensive and anti-neuro-inflammator agents. However, yohimbine is mainly used in treatment for erectile dysfunction. Vindoline used for the development of antidiabetic therapeutics (Almagro *et al.*, 2015). Extracts of different parts of *C. roseus* (leaves, stem, root and flower) having potential against bacterial strains (Muhammad *et al.*, 2009).

Cropping of these plants is taking long time and high cost to extract alkaloids and their metabolites. The extraction of chemical plant products from intact plants has also several inherent problems, including seasonal variations, pests, diseases and inconsistent product quality and yield. Therefore, if an alternative method to plant extraction can be found for producing the active constituents, the need for this slowly growing plant would be reduced, and the pharmaceutical demand could be better satisfied. So many studies and researches were done to discover the other route for production of these compounds (Kargi and Potts, 1991; Yin and Sun, 2011; Ayob and Simarani, 2016).

The products from microbial source are frequently easier and inexpensive (Yin and Sun, 2011). Microorganisms are important sources of natural products

***Corresponding author**

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with activity in drug discovery, agricultural applications and industrial uses (Demain, 1999; Keller *et al.*, 2005; Strobel, 2006; Porras-Alfaro and Bayman, 2011). 40% of new chemical entities (NCEs) collected from 1981 to 2005 have been produced from microorganisms (Khosla, 1997; Clardy and Walsh, 2004; Sieber and Marahiel, 2005).

Endophytes are microorganisms that live inside various parts of plant tissues without causing harm or disease to the hosts (Petrini *et al.*, 1992; Azevedo and Araujo, 2007). These species diversity actually remains unexplored (Srinivasan and Muthumary, 2009). Endopytic microorganisms can be divide into 2 groups; group that don't develop external structures outside the host, while the other group develop external structures as the nodules of nitrogen fixing bacteria and mycorrhizal fungi (Azevedo and Araujo, 2007).

Host plant relationship with endophytes depend on that host plant supply the endophytes with nutrient and habitation for their survival. While endophytes produce number of bioactive compounds for helping the host plants to resist biotic and abiotic external stresses and useful in the host growth (Firáková *et al.*, 2007; Rodriguez *et al.*, 2009).

Terpenoids, alkaloids, phenyl propanoids, aliphatic compounds, poly ketides and peptides were produced by endophytes that have been reported to have antimicrobial activities (Mousa and Raizada, 2013; Pinheiro *et al.*, 2013). Endophytic *Fusarium* species were reported to produce a variety of bioactive secondary metabolites such as fusarubin, javanicin, and marticin which have antimicrobial, insecticidal, phytotoxic, and cytotoxic properties (Kornsakulkarn *et al.*, 2011).

The endophytic *Xylaria* species produce several types of secondary metabolites such as sordaricin, antiplasmodial (+)-phomalactone which act as antifungals and (+)-mycoepoxydiene, and deacetylmycoepoxydiene which are cytotoxic. Endophytic fungi screened from *Garcinia hombroniana* are a rich source of new and bioactive secondary metabolites with different structures (Rukachaisirikul *et al.*, 2013).

Endophytic fungi are able to produce active constituent similar to which produced by their host plants (Gunatilaka, 2006; Zhou *et al.*, 2009). Stierle *et al.*, (1993) found that Taxol and taxane bioactive substance produced by endophytic fungi isolated from *Taxomyces andreanae* is the same as that produced by host plant. Similarly, vinblastine which is a common alkaloid produced by *C. roseus* was detected by using HPLC in the endophytic extract of *Fusarium* sp. isolated from *C. roseus* (Handa, 2015). An endophytic fungus isolated from *Gentiana macrophylla* produces the same bioactive compounds (gentiopicrin or other secoiridoids) so the host plant could be protected from harvest as it is a rare plant (Strobel and Daisy, 2003).

The ability of fungi to grow and produce their metabolites maximally from 2 to 4 weeks let them more economic than their host plants. *C. roseus* associated endophytic fungi could be a promising source of new compounds of industrial and pharmaceutical importance (Akpotu *et al.*, 2017). Thus, this study was carried out to survey the endophytic fungal species isolated from *C. roseus* and screen the alkaloids producing species which have antimicrobial and antitumor activities.

MATERIALS AND METHODS

Plant collection

Fresh plant parts (stem, root and leaves) of *Catharanthus roseus* L. (G) Don. (Apocynaceae) were collected from the Garden in Cairo University, Egypt.

Isolation of endophytic fungi

The plant parts were separated, washed thoroughly with tap water, surface sterilized with 70% ethanol for 1 min, 4% sodium hypochlorite for 3 min and again with 70% ethanol for 1 min, then rinsed twice with sterile distilled water. Samples were dried with sterile filter paper and cut into small pieces with sterile scalpel (Nath and Joshi, 2015). These pieces were then placed on Martin's and Sabouraud's media containing chloramphenicol to suppress bacterial growth. Plates were then incubated at 25-27 °C until the outgrowths of endophytic fungi from the explants were observed. The outgrowths were subcultured to produce pure culture on Cazpex-dox's plates. All isolates were maintained in Cazpex-dox's slants and keep at 4 °C.

Identification of endophytic fungi

The endophytic fungal isolates were identified morphologically and microscopically according to Moubasher (1993).

Alkaloids extraction

Endophytic fungi were inoculated into 250 mL Erlenmeyer flasks containing 100 mL Czapek-Dox's broth media and incubated at 25 °C for 10, 15 and 21 days. Both extracellular and intracellular alkaloids were extracted according to Yin and Chen (2008) with a minor modification. Extracellular alkaloids were extracted with absolute ethanol in water bath at 60 °C for 30 min, then concentrated to about 5-10 mL. The grinded dried mycelia were extracted with ethanol containing 3% HCL at 95 °C for 1.5 h. Filtrations were then done and the filtrates were concentrated to about 5-10 mL. Extracellular and intracellular alkaloids were extracted twice with ethyl acetate after alkalization with NH_4OH to pH 8. Ethyl acetate layers were collected, concentrated and evaporated to dryness. Residues were stored for subsequent analysis.

Qualitative examination of alkaloids

Dragendorff's and Mayer's reagents are used to detect both extracellular and intracellular alkaloids production by changing in the color of the reagents. An orange precipitate with Dragendroff's reagent and a white precipitate with Mayer's reagent indicated alkaloids-producing strains (Chen, 2004; Wu, 2004).

Quantitative analysis of alkaloid

Extracellular and intracellular alkaloids concentrations were determined by absorbance measurement at 280 nm (Kargi and Potts, 1991).

Antimicrobial activity

Antimicrobial activities of the endophytic fungal alkaloid extracts were tested against two Gram positive bacterial species (*Staphylococcus aureus* and *Bacillus subtilis*), one

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Gram-negative bacterial species (*Escherichia coli*) and one fungal (yeast) species (*Candida albicans*). Nutrient agar (NA) media was used for test of bacteria, while candida agar (CA) media was used for fungi. The test organisms were inoculated by streaking over the surface of the sterilized media. The assay was performed using Kirby-Bauer disc diffusion method (NCCLS, 2002, 2003) at fungal extract concentration 5 mg/disc, using DMSO as a solvent. A control test for the solvent was also performed. The petri dishes were incubated at 37 °C for 48 h for bacterial species and at 25 °C for candida for 72 h. Sensitivity was then determined by measuring the mean diameter of the inhibition zones in mm.

In vitro cytotoxicity assay

Analysis was done at National Cancer Institute. *In vitro* cytotoxicity of endophytic fungal extracts isolated from *C. roseus* was performed by using sulforhodamine B (SRB) assay against human breast cancer (MCF-7) and liver cancer (HEPG-2) cell lines using different concentrations of fungal extracts (0, 12.5, 50, 100 µg/mL). The surviving fraction and IC_{50} values were determined (Skehan *et al.*, 1990).

IC_{50} is defined as the concentration which results in a 50% decrease in cell number as compared with that of the control cultures in the absence of an inhibitor (Sun *et al.*, 2011).

GC-MS analysis of fungal alkaloids

Analysis was done at Agricultural Research Center, Giza, Egypt. The analysis was carried out using a GC (Agilent Technologies 7890A) interfaced with a mass selective detector (MSD Agilent 7000) equipped with an apolar Agilent HP- 5ms (5% phenyl methylpolysiloxane) capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium with linear velocity of 1 mL/min. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with reported in the literature (Santanal *et al.*, 2013).

Statistical analysis

The results were expressed as the mean ± standard deviations (mean±SD). Data were analyzed by one-way analysis of variance (ANOVAs) using SPSS statistical program. The differences were considered significant at $p \leq 0.05$. Letters were compared across the same column.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

In the current study, endophytic fungi were isolated from different parts (roots, stems, and leaves) of *C. roseus* using two isolation media; Martin and SDA. Table 1 revealed that Martin media was more suitable for isolation of endophytic fungi (83.92% frequency) than SDA media (16.07% frequency).

A total of 56 endophytic fungal isolates were surveyed from *C. roseus* parts represented by 12 species. *Aspergillus* was the most frequent genus represented by 23 isolates and 3 species, followed by *Fusarium* with 19 isolates and 4 species. The least dominant genus was *Alternaria* constituted one species with 2 isolates. In relation to our study, the fungal genera *Alternaria*, *Fusarium*, *Penicillium*, *Schizophylum* and *Xylaria* were isolated from *C. roseus* as endophytes (Handa, 2015).

With regard to fungal species, *A. fumigatus* and *F. oxysporum* were the most dominant species represented by 11 isolates each with frequency 19.64% of the total isolates (Table 1). *A. niger* came in the second rank with 10 isolates and 17.85% frequency. *Botritis cinerea* and *F. solani* came next with 5 isolates and frequency 8.92%. However, the least frequent species was *F. chlamydosporum* with 1 isolate and 1.78 frequency.

Figure 1 showed that endophytic fungi were more frequent in roots (45 isolates) than both stems and leaves (6 and 5 isolates, respectively). This is convenient with results indicating that endophytic colonization differs with different plant organs (Schulz and Boyle, 2005).

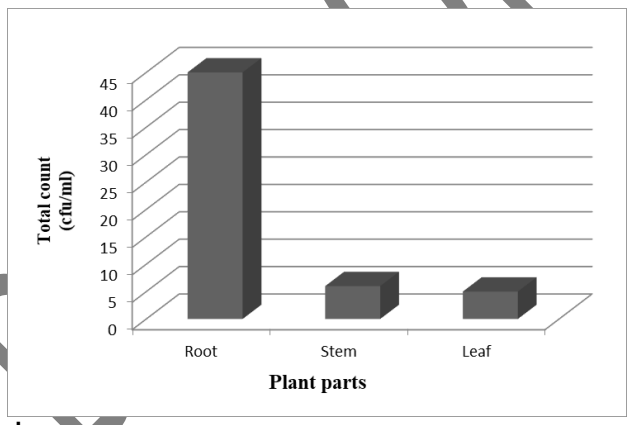

Figure 1: Total count of endophytic fungi isolated from different parts of *C. rosues*.

Alkaloid determination

Both Dragendorff's and Mayer's reagents were used for qualitative screening of both intracellular and extracellular alkaloids produced by the most five dominant endophytic fungal species isolated from *C. roseus*. They were *A. fumigatus*, *A. niger*, *Botrytis* cinera, *F. oxysporum*, *and F. solani.* The results in Table 2 showed that all the five fungal species produce intracellular and extracellular alkaloids. Results also indicated that Dragendorff's reagent was more precise for checking alkaloids production than Mayer's reagent. Yin and Sun (2011) isolated 10 endophytic fungal strains from stem, leaves and roots of *Vinca minor*. The extracts from strain Vm-J_2 only had positive results with both Dragendorff's and Mayer's reagents.

In this study, an experiment was done for quantitative screening of alkaloids in mycelial and filtrate extracts of the most dominant endophytic fungal species isolated from *C. roseus*. Table 3 showed that; by increasing the incubation period the intracellular alkaloid concentrations decreased, while the extracellular ones increased. The most significant total alkaloids productions were by *A. fumigatus* and *A. niger* after 21 days of incubation, while the least one was by *B. cinera* (Figure 2). Different plant parts of *C. roseus* produce intracellular and extracellular indole alkaloids (Abdel-Rahman *et al.*, 2010). The ability of endophytic fungi to produce active components is closely related to their microenvironment (Li *et al.*, 1999; Yin *et al.*, 2009).

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Table 1: Isolation of endophytic fungi from different parts of *Catharanthus roseus*.

Source	Root		Stem		Leaf		TC and F (%)					
Media / Organism	M	S	M	S	M	S	TC M	F (%) M	TC S	F (%) S	TC	F (%)
Alternaria alternata	0	2	0	0	0	0	0	0	2	22.22	2	3.57
Total *Aspergillus* spp.	13		5		5						23	41.07
A. flavus	0	0	0	1	0	1	0	0	2	22.22	2	3.57
A. fumigatus	7	0	0	4	0	0	7	14.89	4	44.44	11	19.64
A. niger	6	0	0	0	4	0	10	21.27	0	0	10	17.85
Botrtis cinerea	4	0	0	1	0	0	4	8.51	1	11.11	5	8.92
Total *Cladosporium* spp.	4		0		0						4	7.14
C. oxysporum	2	0	0	0	0	0	2	4.25	0	0	2	3.57
C. sphaerospermum	2	0	0	0	0	0	2	4.25	0	0	2	3.57
Total *Fusarium* spp.	19		0		0						19	33.92
F. chlamydosporum	1	0	0	0	0	0	1	2.12	0	0	1	1.78
F. moniliforme Var. anthophilum	2	0	0	0	0	0	2	4.25	0	0	2	3.57
F. oxysporum	11	0	0	0	0	0	11	23.40	0	0	11	19.64
F. solani	5	0	0	0	0	0	5	10.63	0	0	5	8.92
Mycelia sterilia	3	0	0	0	0	0	3	6.38	0	0	3	5.35
Total count	43	2	0	6	4	1	47	83.92	9	16.07	56	100

M, matrin medium; S, Sabouraud Dextrose Agar medium (SDA); TC, total count (cfu/mL); F (%), frequency.

Table 2: Qualitative assay of intercellular and extracellular alkaloids produced by endophytic fungal species isolated from *C. roseus*.

Incubation Period & reagent / Organism	10 days				15 days				21 days			
	I		E		I		E		I		E	
	D	M	D	M	D	M	D	M	D	M	D	M
A. Fumgatius	++	++	+	-	**++**	**+**	**++**	**++**	**+**	-	**+**	**+**
A. niger	**++**	**++**	**+**	-	**++**	**++**	**+**	**+**	**+**	**+**	**++**	**++**
B. cinera	**+**	**+**	**+**	-	**++**	**++**	**+**	**+**	**+**	**+**	**+**	**+**
F. oxysporum	**++**	**++**	**+**	**+**	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**
F. solani	++	**++**	**+**	**+**	**+**	**+**	**++**	**++**	**+**	**+**	**++**	**++**

I, intracellular alkaloids; E, extracellular alkaloids; D, Dagrandorff's reagent; M, Mayer's reagent.

Table 3: Quantitative assay of intercellular and extracellular alkaloids produced by endophytic fungal species isolated from *C.* roseus.

Organism	10 Days Incubation		15 Days Incubation		21 Days Incubation	
	I	E	I	E	I	E
A. fumigatus	$22.4^{d} \pm 0.0$	$12^{c} \pm 0.76$	$21.8^{d} \pm 1.2$	$21.2^{d} \pm 1.69$	$21.2^{d} \pm 0.46$	$30.6^{d} \pm 0.68$
A. niger	$25.2^{e} \pm 0.17$	$10.2^{b} \pm 0.3$	$24.8^{e} \pm 0.2$	$11^{b} \pm 0.33$	$24.6^{e} \pm 0.75$	$27.4^{b} \pm 1.2$
B. cinera	$6.2^{a} \pm 0.37$	$5.6^{a} \pm 1.5$	$2.8^{a} \pm 1.45$	8.4 ± 0.6	$1.6^{a} \pm 1.21$	$10.6^{a} \pm 1.63$
F. oxysporum	$21.4^{c} \pm 0.98$	$17.4^{d} \pm 1.8$	$19.6^{c} \pm 2.4$	$19^{c} \pm 1$	$17.8^{c} \pm 1.89$	$29.2^{c} \pm 1.66$
F. solani	$17.6^{b} \pm 0.7$	31.8 ± 0.9	$13.2^{b} \pm 1.28$	$32^{e} \pm 0.3$	$9.2^{b} \pm 0.84$	$32.4^{e} \pm 1.15$

I, Intracellular alkaloids (mg/mL); E, Extracellular alkaloids (mg/m).

Table 4: Antimicrobial activities of filtrate extracts of endophytic fungi isolated from *C. roseus*.

Pathogenic test microorganism	Inhibition zone diameter (mm)					
	A. fumgatis	*A. niger*	*B. cinera*	*F. oxysporum*	*F. solani*	Control
Gram-positive bacteria						
B. subtilis	$7^{b} \pm 1$	$8^{a} \pm 0.0$	$8^{b} \pm 0.0$	$9^{a} \pm 1$	$9^{b} \pm 1$	$0^{a} \pm 0$
S. aureus	$0^{a} \pm 0$	$11^{c} \pm 0.5$	$9^{b} \pm 1.5$	$10^{a} \pm 2$	$9^{b} \pm 0.5$	$0^{a} \pm 0$
Gram-negative bacteria						
E. coli	$6^{b} \pm 0.5$	$8^{a} \pm 1$	$0^{a} \pm 0.0$	$10^{d} \pm 0.0$	$7^{a} \pm 1$	$0^{a} \pm 0$
Yeast						
C. albicans	$9^{c} \pm 0.5$	$9^{b} \pm 0.0$	$11^{c} \pm 1$	$11^{a} \pm 1$	$10^{b} \pm 0.0$	$0^{a} \pm 0$

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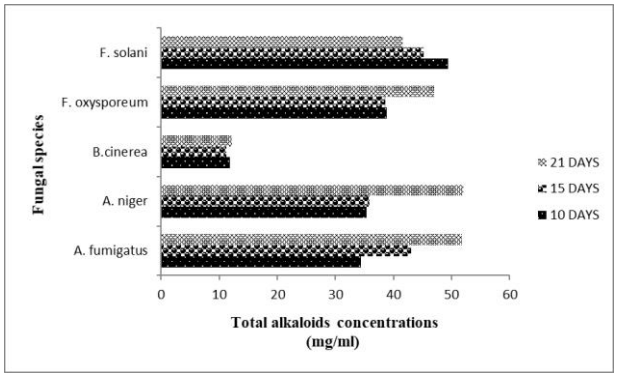

Figure 2: Total alkaloids concentrations after 10, 15 and 21 days incubation periods.

Antimicrobial activity

Antimicrobial activities of mycelial and filtrate extracts of endophytic fungi isolated from *C. roseus* were assayed. The experiment was done against four pathogenic microorganisms; two Gram-positive bacterial species (*B. subtilis* and *S. aureus*), one Gram-negative bacterial species (*E. coli*) and one yeast species (*C. albicans*). As shown in Tables 4 and 5, all fungal extracts had antimicrobial activities against all pathogenic test microorganisms expect filtrate extracts of *A. fumigatus* and *B. cinera* against *S. aureus* and *E. coli*, respectively.
Concerning filtrate extracts (Table 4), *B. subtilis* was highly sensitive to both *F. oxysporum* and *F. solani*, while *S. aureus* was inhibited strongly by *A. niger*. *E. coli* was very susceptible to *F. oxysporum*, while *C. albicans* showed high sensitivity to both *B. cinera* and *F. oxysporum*. Regarding to mycelial extracts (Table 5), *B. cinera* and *F. oxysporum* had the highest significant activities against *B. subtilis*, while *A. niger* was the most effective against *S. aureus*, *E. coli* and *C. albicans*. From the previous results, it was cleared that both *A. niger* and *F. oxysporum* had the most significant antimicrobial activities, while *A. fumigatus* had the least significant activity.

In this field, five endophytic fungi isolated from *C. roseus* showed antibacterial activity when tested against *B. subitilis, E. coli, S. aures* and *S. typhi* and antifungal activity when tested against *A. fumigatius* and *C. albicans* with inhibition zones ranging from 2 to 16 mm (Akpotu *et al.*, 2017).

Different parts of *C. roseus* (leaves, stem, flowers, roots and seeds) showed antibacterial activities against seven bacterial strains (*B. cereus*, *E. coli*, *K. penmonia*, *S. aureus*, *S. sonnei*, *S. paratypyi* and *S. typhi*) with different sensitivities (Jayakumar *et al.*, 2010). Shanmugaraju and Bhakyaraj (2016) tested the antibacterial activity of *C. roseus* leaf extract. They observed that *Staphylococcus* sp was more susceptible than *E. coli*, *Pseudomonas* sp and *Streptococcus* sp. This was agreed with the hypotheses that endophytic fungi are having capacity to produce bioactive substances and secondary metabolites similar to that produced by their host plants (Gunatilaka, 2006; Zhou *et al.*, 2009). Endophytes should produce metabolites to compete first with other endophytes and then with pathogens to colonize the host (Schulz *et al.*, 2002). Most of these metabolites display antimicrobial and cytotoxic activities (Sun *et al.*, 2011). So, endophytic fungi act as an important source for developing drug discovery process (Strobel, 2003; Firàkovà *et al.*, 2007; Debbab *et al.*, 2011).

***In vitro* cytotoxicity assay**

The *in vitro* cytotoxicity assay of extracts of endophytic fungal species (which had the highest antimicrobial activities) isolated from *C. roseus* was determined against breast cancer (MCF-7) and liver cancer (HEPG-2) human cell lines by using SRB assay method. As shown in Table 6, it was obvious that both fungal extracts showed cytotoxic effects on the two cell lines. *A. niger* extract displayed the highest significant cytotoxicity against MCF-7 cell line with IC_{50} value of 42.1 µg/mL, while the least cytotoxicity was exhibited by *F. oxysporum* against MCF-7 cell line with IC_{50} value of 66.9 µg/mL.

Similarly, mycelial extract of the fungus *Nigrospora sphaerica* isolated from *C. roseus* produced vinblastine with a concentration higher than produced by the plant leaf extract. Vinblastine produced by *N. sphaerica* had a higher cytotoxic activity when using MTT assay against breast cell line cancer MDA-MB 231 (Ayob *et al.*, 2017). Different extracts (methanol, ethanol, chloroform, ethyl acetate and acetone) of aerial parts of *C. roseus* showed *in vitro* cytotoxicity against MCF (breast cancer) cell lines by using MTT assay method (Ruskin and Aruna, 2014). This means that *C. roseus* had activities similar to their endophytic fungi, so further researches are needed to study the active constituent of both plants and their endophytes.

GC-MS analysis of fungal alkaloids

Elucidation of the alkaloids in mycelial and filtrate extracts of *A. niger* was carried out using gas chromatography mass spectroscopy (GC-MS). As shown in Table 7, 11 alkaloid compounds were detected in both mycelial and filtrate extracts of *A. niger* with different retention times. They were Thebaine, (16s,19Z)-Isositsirikine, Pleiocarpamine, Aspidofractinine, Dihydrovallesiachot-amine, Vincamine, Ajmaline, (-)-Vincadifformine, 20-Cycloaspidospermidine-3-carboxylic acid, 1-formyl-8-oxo-, methyl ester, (2α,3β,5α,12β,19α,20R), Reserpine and Epiibogamine. Percentages of Thebaine, Aspidofractinine, Vincamine and Epiibogamine alkaloid compounds in mycelial extracts were higher than in filtrate extracts. In contrast, percentages of (16s,19Z)-Isositsirikine, Pleiocarpamine, Dihydrovallesiachotamine, Ajmaline, (-)-Vincadifformine, 2,20-Cycloaspidospermidine-3-carboxylic acid, 1-formyl-8-oxo-, methyl ester, (2α,3β,5α,12β,19α,20R) and Reserpine were more in filtrate extracts than in mycelial extracts.

It was clearly observed that endophytic fungi and their host plants may produce similar bioactive compounds. This leads to hypothesis that the bioactivity of the host plant may be due to metabolites produced by the endophytic microorganisms.

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Table 5: Antimicrobial activities of mycelial extracts of endophytic fungi isolated from *C. roseus*.

Pathogenic test microorganism	Inhibition zone diameter (mm)					
	A. fumgatis	*A. niger*	*B. cinera*	*F. oxysporum*	*F. solani*	Control
Gram-positive bacteria						
B. subtilis	$7^a \pm 1$	$10^a \pm 0.0$	$11^c \pm 1$	$11^b \pm 1.5$	$9^a \pm 1.5$	$0^a \pm 0$
S. aureus	$9^b \pm 0.0$	$10^a \pm 0.5$	$9^b \pm 0.5$	$9^a \pm 0.0$	$8^a \pm 1.5$	$0^a \pm 0$
Gram-negative bacteria						
E. coli	$7^a \pm 0.5$	$10^a \pm 1$	$6^a \pm 0.5$	$7^a \pm 1$	$9^a \pm 0.5$	$0^a \pm 0$
Yeast						
C. albicans	$5^a \pm 0.5$	$11^a \pm 0.0$	$9^b \pm 1$	$9^a \pm 0.0$	$8^a \pm 0.5$	$0^a \pm 0$

Table 6: Cytotoxicity assay of endophytic fungal extracts against MCF-7 and HEPG-2 human cell lines.

Conc. (µg/mL)	Surviving fraction			
	A. niger		*F. oxysporum*	
	MCF-7	HEPG-2	MCF-7	HEPG-2
0.0	$1.000^a \pm 0.000$	$1.000^a \pm 0.000$	$1.000^a \pm 0.000$	$1.000^a \pm 0.000$
12.5	$0.844^b \pm 0.001$	$0.867^b \pm 0.002$	$1.000^a \pm 0.000$	$1.007^b \pm 0.002$
25	$0.856^c \pm 0.002$	$0.806^c \pm 0.001$	$0.835^b \pm 0.002$	$0.926^c \pm 0.001$
50	$0.296^d \pm 0.001$	$0.481^d \pm 0.001$	$0.593^c \pm 0.001$	$0.541^d \pm 0.001$
100	$0.349^e \pm 0.002$	$0.496^e \pm 0.002$	$0.336^d \pm 0.001$	$0.389^e \pm 0.001$
IC_{50} (µg/mL)	42.1	48.2	66.9	61.9

Table 7: GC-MS analysis of alkaloids in mycelial and filtrate extracts of *A. niger*.

Alkaloid	**Retention time (min)**	**Area sum% (Filtrate)**	**Area sum% (Mycelia)**
Thebaine	4.4	0.42	1.67
(16s,19Z)-Isositsirikine	7.7	0.75	0.53
Pleiocarpamine	9.59	1.92	0.71
Aspidofractinine	13.6	0.4	0.58
Dihydrovallesiachotamine	14.3	1.48	0.65
Vincamine	16.92	0.39	4.37
Ajmaline	19.52	6.86	0.47
Vincadifformine(-)-	19.78	1.96	1.27
2,20-Cycloaspidospermidine-3-carboxylic acid, 1-formyl-8-oxo-, methyl ester, (2α,3β,5α,12β,19α,20R)	20.44	5.59	1.07
Reserpine	20.6	0.94	0.76
Epiibogamine	21.5	36.44	44.56

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REFERENCES

Abdel-Rahman, T. M., Kapiel, T., Ibrahim, D. M. and Ali, E. A. (2010). Elicitation of alkaloids by biotic and abiotic stress factors in *Catharanthus roseus* L. (G) Don. *Egyptian Journal of Botany* **207-224.**

Akpotu, M. O., Eze, P. M., Abba, C. C., Umeokoli, B. O., Nwachukwu, C. U., Okoye, F. B. C. and Esimone, C. O. (2017). Antimicrobial activities of secondary metabolites of endophytic fungi isolated from *Catharanthus roseus*. *Journal of Health Sciences* **7(1), 15-22.**

Almagro, L., Fernández-Pérez, F. and Pedreño, M. (2015). Indole alkaloids from *Catharanthus roseus*: Bioproduction and their effect on human health. *Molecules* **20(2), 2973-3000.**

Ayob, F. W. and Simarani, K. (2016). Endophytic filamentous fungi from a *Catharanthus roseus*: Identification and its hydrolytic enzymes. *Saudi Pharmaceutical Journal* **24(3), 273-278.**

Ayob, F. W., Simarani, K., Zainal Abidin, N. and Mohamad, J. (2017). First report on a novel *Nigrospora sphaerica* isolated from *Catharanthus roseus* plant with anticarcinogenic properties. *Microbial Biotechnology* **10(4), 926-932.**

Azevedo, J. L. and Araujo, W. L. (2007). Diversity and applications of endophytic fungi isolated from tropical plants. In: Fungi multifaceted microbes. Ganguli, B. N. and Deshmukh, S. K. (eds.). Anamaya, New Delhi.

Balaabirami, S. and Patharajan, S. (2012). In vitro antimicrobial and antifungal activity of *Catharanthus roseus* leaves extract against important pathogenic organisms. *International Journal of Pharmacy and Pharmaceutical Sciences* **4(3), 487-490.**

Chen, Q., Miao, J. H. and Ma, J. (1995). Determination of vincamine in tissue culture from *Vinca minor L*. by TLC scanning method. *Journal of China Pharmaceutical University* **6, 350-352.**

Chen, Y. G. (2004). Phytochemical Components. Chemical Industry Press. Beijing, China. **pp. 275.**

Chevallier, A. (1996). The Encyclopedia of Medicinal Plants. Dorling Kindersley, London.

Clardy, J. and Walsh, C. (2004). Lessons from natural molecules. ***Nature* 432, 829-837.**

Debbab, A., Ahy, A. H. and Proksch, P. (2011). Bioactive secondary metabolites from endophytes and associated marine derived fungi. *Fungal Diversity* **49, 1-12.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Demain, A. L. (1999). Pharmaceutically active secondary metabolites of microorganisms. *Application Microbiology Biotechnol*ogy **52, 455-463.**

Firáková, S., Šturdíková, M. and Múcková, M. (2007). Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* **62, 251-257.**

Gajalakshmi, S., Vijayalakshmi, S. and Devi, R. V. (2013). Pharmacological activities of *Catharanthus roseus*: a perspective review. *International Journal of Pharma and Bio Sciences* **4(2), 431-439.**

Grellier, P., Sinou, V., Garreau-de Loubresse, N., Bylen, E., Boulard, Y. and Schrevel, J. (1999). Selective and reversible effects of vinca alkaloids on *Trypanosoma cruzi* epimastigote forms: Blockage of cytokinesis without inhibition of the organelle duplication. *Cell Motil Cytoskeleton* **42, 36-47.**

Gunatilaka, A. A. (2006). Natural products from plant-associated microorganisms: distribution, structural diversity, bioactivity, and implications of their occurrence. *Journal of Natural Products* **69(3), 509-526.**

Handa, G. (2015). Isolation and screening of Vinblastine from Endophytic Fungi. M.Sc. Thesis. Patiala, India.

Jaleel, C. A., Gopi, A. R., Lakshmanan, G. M. and Panneerselvam, R. (2006). Triadimefon induced changes in the antioxidant metabolism and ajmalicine production in *Catharanthus roseus* (L.) *G.* Don. *Plant Science* **171, 271-276.**

Jayakumar, D., Mary, S. J. and Santhi, R. J. (2010). Evaluation of antioxidant potential and antibacterial activity of *Calotropis gigantea* and *vinca rosea* using in vitro model. *Indian Journal of Science and Technology* **3(7), 720-723.**

Kargi, F. and Potts, P. (1991). Effect of various stress factors on indole alkaloid formation by *C. roseus* (Periwinkle) cells. *Enzyme and Microbial Technology* **13(9), 760-763.**

Karimi, E. and Jaafar, H. Z. (2011). HPLC and GC–MS determination of bioactive compounds in microwave obtained extracts of three varieties of *Labisia pumila* Benth. *Molecules* **16(8), 6791-6805.**

Keller, N. P., Turner, G. and Bennett, J. W. (2005). Fungal secondary metabolism from biochemistry to genomics. *Nature. Rev. Microbiol* **3(12), 937-947.**

Khosla, C. (1997). Harnessing the biosynthetic potential of modular polyketide synthases. *Chem. Rev*. **97, 2577-2590.**

Kornsakulkarn, J., Dolsophon, K., Boonyuen, N., Boonruangprapa, T., Rachtawee, P., Prabpai, S., Kongsaeree, P. and Thongpanchang, C. (2011). Dihydronaphthalenones from endophytic fungus *Fusarium sp.* BCC14842. *Tetrahedron* **67, 7540-7547.**

Kumar, A., Malik, R., Giri, P., Parveen, N., Shweta, (2015). Up-to-date review on therapeutic interior of catharanthus roseus; for anticancer and antidiabetic activities. *Journal of Phytomedicine* **1(1), 1-13.**

Li, H. Y., Zeng, S. R. and Zhang, L. Q. (1999). The diversity of the endophytic fungi isolated from the underground stems of *Podophyllum hexandrum* (Royle) Ying and the selection of a valuable isolate. *Southwest China Journal of Agricultural Sciences* **4, 123-125.**

McAlpine, J. B., Bachmann, B. O., Piraee, M., Tremblay, S., Alarco, A. M., Zazopoulos, E., and Farnet, C. M. (2005). Microbial genomics as a guide to drug discovery, structural elucidation: ECO02301, a novel antifungal agent, as an example. *Journal of Natural Products* **68, 493-496.**

Mohan, K., Jeyachandran, R. and Deepa, (2012). Alkaloids as anticancer agents. *Annals of Phytomedicine* **1(1), 46-53.**

Moubasher, A. H. (1993). Soil Fungi in Qatar and Other Arab Countries. The Center for Scientific and Research, University of Qatar, Qatar.

Mousa, W. K. and Raizada, M. N. (2013). The diversity of anti-microbial secondary metabolites, fungal endophytes: an interdisciplinary perspective. *Frontiers in Microbiology* **4(65), 1-18.**

Muhammad, L. R., Muhammad, N., Tanveer, A. and Baqir, S. N. (2009). Antibacterial activity of different extracts from the *Catharanthus roseus*. *Clinical and Experimental Medical Journal* **3(1), 81-85.**

Nath, A. and Joshi, S. R. (2015). Ultrastructural effect on mastitis pathogens by extract of endophytic fungi associated with ethnoveterinary plant, *Hibiscus sabdariffa* L. *Journal of Microscopy and Ultrastructure* **3(1), 38-43.**

National Committee for Clinical Laboratory Standards., (2002). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi: Proposed Standard M 38-A. NCCLS, Wayne, PA, USA.

National Committee for Clinical Laboratory Standards., (2003). Method for Antifungal Disk Diffusion Susceptibility Testing of Yeast: Proposed Guideline M44-P. NCCLS, Wayne, PA, USA.

Petrini, O., Sieber, T. N., Toti, L. and Viret, O. (1992). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Natural Toxins* **1(3), 185-196.**

Pinheiro, E. A., Carvalho, J. M., Dos Santos, D. C., Feitosa, A., Marinho, P. S. and Guilhon, G. M. (2013). Antibacterial activity of alkaloids produced by endophytic fungus *Aspergillus* sp. EJC08 isolated from medical plant *Bauhinia guianensis*. *Natural Product Research* **27(18), 1633-1638.**

Porras-Alfaro, A. and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Annual Review of Phytopathology* **49, 291-315.**

Rodriguez, R. J., White, J. F., Arnold, A. E. and Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist*. **182(2), 314-330.**

Rukachaisirikul, V., Buadama, S., Phongpaichit, S. and Sakayaroj, J. (2013). Amide, cyclohexenone, and cyclohexenonee sordaricin derivatives from the endophytic fungus *Xylaria plebeja* PSU-G30. *Tetrahedron* **69, 10711-10717.**

Ruskin, R. S. and Aruna, S. R. (2014). *In-vitro* and *in-vivo* antitumor activity of *Catharanthus roseus*. *International Research Journal of Pharmaceutical and Applied Sciences* **4(6), 1-4.**

Santanal, P. M., Mirada, M., Payrol, J. A., Silva, M., Hernàndez, V. and Peralta, E. (2013). Gas chromatography-mass spectrometry study from the leaves fractions obtained of *Vernonanthura patens* (Kunth) H. Rob. *International Journal of Organic Chemistry* **3, 105-109.**

Schulz, B. and Boyle, C. (2005). The endophytic continuum. *Mycological Research*. **6, 661-686.**

Schulz, B., Boyle, C., Draeger, S., Römmert, A. K. and Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* **106, 996-1004.**

Shanmugaraju, V. and Bhakyaraj, R. (2016). Antimicrobial potential activity of leaf extracts of *Catharanthus roseus* against human pathogens under

CORRECTED PROOF

ISSN (print): 1823-8262, ISSN (online): 2231-7538

laboratory conditions. *International Journal of Current Research in Biology and Medicine* **1(1), 35-51.**

Sieber, S. A. and Marahiel, M. A. (2005). Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. *Chemical.Reviews* **105, 715-738.**

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., … Boyd, M. R. (**1990).** New colorimetric cytotoxicity assay for anticancer-drug screening. *Journal of the National Cancer Institute* **82(13), 1107-1112.**

Srinivasan, K. and Muthumary, J. (2009). Taxol production from *Pestalotiopsis* sp and endophytic fungus isolated from *Catharanthus roseus. Journal of Ecobiotechnology* **1(1), 028-031.**

Stierle, A., Strobel, G. A. and Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*. *Science* **260, 214-216.**

Strobel, G. (2003). Endophytes as source of bioactive products. *Microbes and Infection* **5, 535-544.**

Strobel G. (2006). Harnessing endophytes for industrial microbiology. *Current Opinion in Microbiology* **9, 240-244.**

Strobel, G. and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* **67(4), 491-502.**

Sun, Z., Zhang, M., Zhang, J. and Feng, J. (2011). Antifungal and cytotoxic activities of the secondary metabolites from endophytic fungus *Massrison* sp. *Phytomedicine* **18, 859-862.**

Wu, L. J. (2004). Natural Pharmaceutical Chemistry. People's Medical Publishing House. Beijing, China. **pp. 376-377.**

Yamamoto K, Takahashi K, Mizuno H, Anegawa A, Ishizaki K, Fukaki H, Ohnishi M, … Mimura T. (2016). Cell-specific localization of alkaloids in *Catharanthus roseus* stem tissue measured with Imaging MS and Single-cell MS. *Proc Natl Acad Sci USA* **113(14), 3891-3896.**

Yin, H. and Chen, J. L. (2008). Sipeimine-producing endophytic fungus isolated from *Fritillaria ussuriensis*. *Zeitschrift für Naturforschung C* **63(11-12), 789-793.**

Yin, H. and Sun, H. (2011). Vincamine-producing endophytic fungus isolated from *Vinca minor*. *Phytomedicine* **18, 802-805.**

Yin, H., Zhao, Q., Sun, F. M. and An, T. (2009). Gentiopicrin-producing endophytic fungus isolated from *Gentiana macrophylla*. *Phytomedicine* **16(8), 793-797.**

Zhou, L., Zhao, J., Xu, L., Huang, Y., Ma, Z., Wang, J. and Jiang, W. (2009). Antimicrobial compounds produced by plant endophytic fungi. In: Fungicides: Chemistry, Environmental Impact and Health Effects. De Costa, P. and Bezerra, P. (eds.). Nova Science Publishers, New York. **pp. 91-11.**

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