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# Cytotoxic and antibacterial naphthoquinones from an endophytic fungus, *Cladosporium* sp.

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ABSTRACT

*Objective:* Endophytes have the potential to synthesize various bioactive secondary metabolites. The aim of the study was to find new cytotoxic and antibacterial metabolites from endophytic fungus, *Cladosporium* sp. isolated from the leaves of *Rauwolfia serpentina* (L.) Benth. ex Kurz. (Fam: Apocyanaceae).
*Materials and methods:* The endophytic fungus was grown on potato dextrose agar medium and extracted using ethyl acetate. Secondary metabolites were isolated by chromatographic separation and re-crystallization, and structures were confirmed by $^{1}$H NMR, $^{13}$C NMR and mass spectroscopic data. The cytotoxicity was determined by WST-1 assay and brine shrimp lethality bioassay, while antibacterial activity was assessed by disc diffusion method.
*Results:* Two naphthoquinones, namely anhydrofusarubin (**1**) and methyl ether of fusarubin (2), were isolated from *Cladosporium* sp. The isolated compounds **1** and **2,** by WST-1 assay against human leukemia cells (K-562) showed potential cytotoxicity with $IC_{50}$ values of 3.97 μg/mL and 3.58 μg/mL, respectively. Initial screening of crude ethyl acetate extract and column fractions F-8 and F-10 exhibited noticeable cytotoxicity to brine shimp nauplii with $LC_{50}$ values of 42.8, 1.2 and 2.1 μg/mL, respectively. Moreover, the isolated compound 2 (40 μg/disc) showed prominent activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus megaterium* with an average zone of inhibition of 27 mm, 25 mm, 24 mm and 22 mm, respectively and the activities were compared with kanamycin (30 μg/disc).
*Conclusion:* Our findings indicate that anhydrofusarubin (**1**) and methyl ether of fusarubin (**2**) might be useful lead compounds to develop potential cytotoxic and antimicrobial drugs.


## 1. Introduction

Secondary metabolites having cytotoxic properties have the potential to explore as anticancer and antibacterial drugs. Until now many cytotoxic agents including paclitaxel (also known as Taxol) [1] have been isolated from endophytes. An endophytic fungus is an endosymbiont that lives within a plant [2,3]. Many endophytes have the potential to synthesize various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases [4–8]. However, there is a need for search of new antibacterial and cytotoxic agents that are highly effective. The search is driven by the development of resistance in infectious bacteria and cancer cells to existing drugs.

Occasionally, endophytes that produce host plant secondary metabolites with therapeutic value or potential have been discovered; some examples include podophyllotoxin [9,10], deoxypodophyllotoxin [11], camptothecin and structural analogs [12–15], hypericin and emodin [16,17], and azadirachtin [18]. Hence, endophytic fungi may be considered as important sources for the discovery of lead compounds for new drugs [19]. It is worth mentioning that endophytic fungi produce novel compounds with diverse chemical skeletons and biological activities [20–22].

To search for new cytotoxic and antibacterial compounds, an endophytic fungus, *Cladosporium* sp. was isolated from the leaves of *Rauwolfia serpentina* (Fam: Apocyanaceae). Some of the important metabolites from *Cladosporium* sp include *p*-methylbenzoic acid and peroxyergosterol [23], cytotoxic aspernigrin A [24], antifungal

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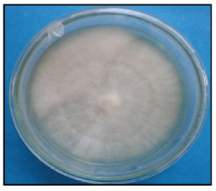

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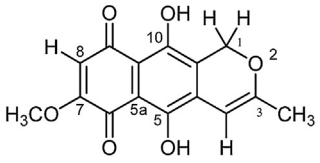

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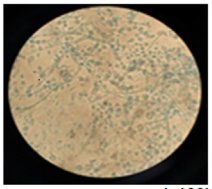


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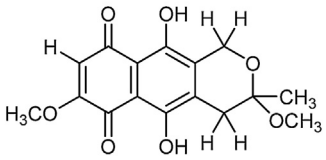

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**Fig. 1.** Chemical structures of anhydrofusarubin (**1**) and methyl ether of fusarubin (**2**) isolated from *Cladosporium* sp. (**A**. Macroscopic view and **B**. Microscopic view of the cultured dish).

phleichrome [19], macrolide metabolites: pandangolides 2, 3 and 4, cladospolide B, and isocladospolide B and antimicrobial furan carboxylic acids: Sumiki's acid and its new derivative, acetyl Sumiki's acid [25], cladosporin, isocladosporin, 5′-hydroxyasperentin, and cladosporin-8-methyl ether [26], and aconitine [27].

As part of our continuing investigations to find new cytotoxic and antibacterial metabolites from endophytic fungi, we investigated the ethyl acetate extracts of culture of the endophytic fungus, *Cladosporium* sp., grown on potato dextrose agar medium. From these extracts, two naphthoquinones (Fig. 1) were isolated in pure form by a combination of repeated chromatography and crystallization and characterized by spectroscopic methods. Cytotoxicity was evaluated by brine shrimp lethality assay and WST-1 assay, while antibacterial activity was assessed by disc diffusion method. Crude fungal extract as well as several column fractions showed prominent cytotoxicity and antibacterials activities. Isolated pure compound **2** exhibited significant antibacterial and anticancer activities, whereas compound **1** showed prominent anticancer activity.

## 2. Materials and methods

### 2.1. General procedures

NMR studies of the isolated pure compounds were carried out using deuterated chloroform and the δ values for $^{1}H$ and $^{13}C$ NMR spectral data were referred to the residual nondeuterated solvent signals. The $^{1}H$ and $^{13}C$ NMR spectral data were obtained using a Varian Unity 500 spectrometer. ESIMS was recorded on a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu LC/MS-IT-TOF). The structures of the compounds were identified by spectroscopic analysis and comparison of NMR data with published literature.

### 2.2. Isolation of secondary metabolites

*Cladosporium* species, internal strain no. RSBE-3, which had been isolated following surface sterilization from the barks of the plant *Rauwolfia serpentina* was cultivated at room temperature for 21d on potato dextrose agar (PDA) medium. The culture medium was extracted three times with ethyl acetate to obtain the crude extract (3.0 g). The crude extract was subjected to column chromatography for fractionation on silica gel by using gradients of petroleum ether/dichloromethane, then dichloromethane, followed by a gradient of dichloromethane/methanol, and finally methanol to afford a total of 22 fractions. These fractions were screened by TLC on silica gel under UV light in both short (254 nm) and long (365 nm) wavelengths and by spraying with vanillin-$H_2SO_4$ spray reagents.The column fraction of petroleum ether/75% dichloromethane was subjected to column chromatography for further fractionation. After crystallization from petroleum ether/dichloromethane (50%) gave fine needles of compound **1** (5.62 mg). The column fraction of dichloromethane/methanol (50%) was subjected to column chromatography for further fractionation. After crystallization from dichloromethane/methanol (1.5%) gave fine needles of compound **2** (9.46 mg).

#### 2.2.1. Compound 1 (anhydrofusarubin)

It appeared as dark violet spot on the TLC plate. It is soluble in dichloromethane, chloroform and sparingly soluble in methanol. Yield 5.62 mg. $R_f$ 0.43 (toluene/5% EtOAc); $^{1}H$ NMR (500 MHz, $CDCl_3$): δ 1.98 (3H, s, $CH_3$-3), 3.88 (3H, s, $OCH_3$-7), 5.16 (2H, s, $CH_2$-1), 5.92 (1H, s, H-4), 6.11 (1H, s, H-8), 12.57 (1H, s, OH-5), 12.97(1H, s, OH-10),$^{13}C$ NMR (125 MHz, $CDCl_3$): δ 20.1 (C-11), 56.6 (C-12), 62.9 (C-1), 94.6 (C-4), 107.9 (C-9a), 109.9 (C-8), 110.9 (C-5a), 122.7 (C-10a), 132.9 (C-4a), 157.6 (C-10), 157.6 (C-5), 159.9 (C-7), 161.5 (C-3), 177.8 (C-6), 182.9 (C-9).ESIMS: $m/z$ = 289 $[M+H]^+$.

#### 2.2.2. Compound 2 (methyl ether of fusarubin)

It appeared as dark quenching spot on the TLC plate. It is soluble in dichloromethane, chloroform and sparingly soluble in methanol. Yield 9.46 mg. $R_f$ 0.44 (toluene/20% EtOAc); $^{1}H$ NMR (500 MHz, $CDCl_3$): $\delta$ 1.53 (3H, s, $CH_3$-11), 2.65 (1H, dt, $J_{4,4}$ = 18.0 Hz, $J_{4,1}$ = 2.0 Hz, H-4), 2.99 (1H, dd, $J_{4,4}$ = 18.0 Hz, $J_{4,1}$ = 1.5 Hz, $CH_3$-4), 3.30 (3H, s, $OCH_3$-12), 3.91 (3H, s, $OCH_3$-13), 4.54 (1H, dt, $J_{1,1}$ = 17.8 Hz, $J_{1,4}$ = 2.7 Hz, H-1), 4.85 (1H, dd, $J_{1,1}$ = 17.8 Hz, $J_{1,4}$ = 1.5 Hz H-1), 6.15 (1H, s, H-8), 12.63 (1H, s, OH-5), 12.91 (1H, s, OH-10),$^{13}C$ NMR (125 MHz, $CDCl_3$):$\delta$ 22.8 (C-11), 33.0 (C-4), 48.9 (C-2), 56.7 (C-13), 58.7 (C-1), 96.8 (C-3), 107.5 (C-9a), 109.6 (C-5a), 109.7 (C-8), 132.9 (C-4a), 137.2 (C-10a), 157.2 (C-7), 160.7 (C-5), 160.7 (C-10), 178.2 (C-6), 184.7 (C-9). ESIMS: $m/z$ = 321 $[M+H]^+$.

### 2.3. Cytotoxic activity test by WST-1 assay

Inhibition of cancer cell growth for compounds **1** and **2** was determined by WST-1 (4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2*H*-5-tetrazolio)-1,3-benzene disulfonate) assay [28,29] using Triton X-100 and hydrogen peroxide ($H_2O_2$) solutions as positive controls. In brief, the human leukemia cell (K-562) was cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum at 37 °C in humidified air containing 5% $CO_2$. The cells were distributed at proper density ($2 \times 10^4$ cells/mL) in 96-well plates and compounds (0.5–30 μg/mL) were added. The suspensions were incubated for 5 days under above condition. On the addition of WST-1 solution (1.0 mg/mL of stock solution, 50 μL each), the suspensions were further incubated for 4 h under the same condition. After removing the supernatant with microplate washer, 150 μL of DMSO was added to dissolve formazen. The absorbance was measured at 480 nm with a microplate reader and $IC_{50}$ values were calculated.

### 2.4. Brine shrimp lethality bioassay

The cytotoxicity of the crude fungal extract and eight column fractions (F-1, F-2, F-3, F-4, F-5, F-8, F-10, and F-13) were tested following the methods as described previously [30,31]. Briefly, the test samples were dissolved in DMSO and then diluted as 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 μg/mL following a serial dilution procedure. A series of test tubes containing 10 shrimps in simulated brine water (5 mL) were prepared and marked properly, and then each of test solutions was added to the marked test tubes

and incubated at room temperature for 24 h. The lethality ($LC_{50}$ values) of the test samples was determined by drawing curves against percentage the shrimps killed versus the logarithm of test sample concentration. Vincristine sulphate, an anticancer drug, was used as standard to compare the cytotoxicity of the crude extract and column fractions.

### 2.5. Antibacterial activity assay

Column fractions and compound **2** was tested for antibacterial activity by disc diffusion method [32,33]. Gram positive bacterial strains such as *Staphylococcus aureus* and *Bacillus megaterium*; and Gram negative bacterial strains such as *Escherichia coli* and *Pseudomonas aeruginosa* were used for the experiment. Bacterial strains were collected as pure cultures from the Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR). Compound **2** was tested at 40 μg/disc whereas column fractions were tested at 100 μg/disc and the zone of inhibition were compared with that of kanamycin (30 μg/disc) and ketoconazole (30 μg/disc).

## 3. Results and discussion

Large scale cultivation of endophytic fungus, *Cladosporium* sp. on PDA medium followed by extraction with ethyl acetate and repeated chromatographic separation with crystallization process yielded two naphthoquinones, namely anhydrofusarubin (**1**) and methyl ether of fusarubin (**2**). This is the first report of their isolation from the *Cladosporium* sp. The structures were confirmed by $^1$H NMR, $^{13}$C NMR and mass spectroscopic data.

Compound **1** was isolated as violet crystal from a combined column fractions with the eluent system petroleum ether/dichloromethane (50%). It was appeared as a purple spot on the TLC plate and is soluble in dichloromethane, chloroform and sparingly soluble in methanol. $^{13}$C NMR spectrum (125 MHz, $CDCl_3$) of **1** displayed 15 carbon resonances. The resonance at **δ** 1.98 ppm in the $^1$H NMR (500 MHz, $CDCl_3$) and at $\delta$ 20.1 ppm in the $^{13}$C NMR spectra could be attributed for one methyl group. The reasonance at $\delta$ 3.88 ppm in the $^1$H NMR and at $\delta$ 56.6 ppm in the $^{13}$C NMR spectra proved the presence of a methoxy group in **1**. The resonances at $\delta$ 5.92 ppm and $\delta$ 6.11 ppm in the $^1$H NMR and $\delta$ 94.6 ppm and $\delta$ 109.9 ppm in the $^{13}$C NMR spectra could be attributed to two-olefenic protons. The presence of one two proton singlet at $\delta$ 5.16 ppm in the $^1$H NMR and at $\delta$ 62.9 ppm in the $^{13}$C NMR spectra indicated the presence of two equivalent aliphatic protons.The presence of two sharp proton singlets at $\delta$ 12.57 ppm and $\delta$ 12.97 ppm in $^1$H NMR spectrum could be attributed to two phenolic chelated hydroxyl groups. The relatively deshielded nature of these hydroxyl groups indicated that each of them might form intramolecular hydrogen bonds with any lone electron pairs of a functional group. The resonance at $\delta$ 177.8 ppm and $\delta$ 182.9 ppm could be attributed to two carbonyl carbons. The relatively shielded nature of these two carbonyl carbons indicated their belongings to a quinone system fused to the aromatic ring. So it is now obvious that in **1**, the carbonyl groups of quinone system formed two intramolecular hydrogen bonds with the two deshielded phenolic hydroxyl groups. The gross structure of **1** was confirmed by the mass spectrum with $[M+H]^+$ at $m/z = 289$, suggesting the molecular formula $C_{15}H_{12}O_6$, in agreement with the NMR spectra. Finally, the structure of **1** was confirmed as anhydrofusarubin by comparison with the published NMR data of isolated anhydrofusarubin from the fungus *Fusarium solani* [34,35].

Compound **2** was also obtained as orange colored crystals from a sub-column fraction with the eluent system dichloromethane/methanol (1.5%) and appeared as orange spot on the TLC plate. It is soluble in dichloromethane, chloroform and sparingly soluble in methanol. The $^{13}$C NMR spectrum (125 MHz, $CDCl_3$) of **2** displayed 16 carbon resonances. The resonance at $\delta$ 1.53 ppm in the $^1$H NMR (500 MHz, $CDCl_3$) and at $\delta$ 22.80 ppm in the $^{13}$C NMR spectra could be attributed for one methyl group. The presence of reasonance at $\delta$ 3.30 ppm and $\delta$ 3.91 ppm in the $^1$H NMR and at $\delta$ 48.9 ppm and $\delta$ 56.7 ppm in the $^{13}$C NMR spectra proved the presence of two methoxy groups in **2**. The resonances at $\delta$ 6.15 ppm in the $^1$H NMR and at $\delta$ 109.7 ppm in the $^{13}$C NMR spectra could be attributed to one-olefenic proton. The presence of one one-proton doublet of triplet at $\delta$ 4.54 ppm and another one proton doublet of doublet at 4.85 ppm in the $^1$H NMR spectrum could be attributed to two aliphatic protons. The larger coupling constant ($J$ = 17.8 Hz) of these aliphatic protons indicated that they are geminal. The presence of another one-proton doublet of triplet at $\delta$ 2.65 ppm and another double of doublet at $\delta$ 2.99 ppm in the $^1$H NMR spectrum could be attributed to two aliphatic protons. The larger coupling constant ($J$ = 18.0 Hz) of these aliphatic protons indicated that they are geminal. The coupling pattern and the smaller coupling constant (2.0 Hz) indicated that the former geminal protons also did long range coupling with the latter geminal protons. The presence of two sharp proton singlets at $\delta$ 12.63 ppm and $\delta$ 12.91 ppm in $^1$H NMR spectrum could be attributed to two phenolic chelated hydroxyl groups. The relatively deshielded nature of these hydroxyl groups indicated that each of them might form intramolecular hydrogen bonds with any lone electron pairs of a functional group. The resonance at $\delta$ 178.2 ppm and $\delta$ 184.7 ppm could be attributed to two carbonyl carbons. The relatively shielded nature of these two carbonyl carbons indicated their belongings to a quinone system fused to the aromatic ring. Hence, it is obvious that in **2**, the carbonyl groups of quinone system formed two intramolecular hydrogen bonds with the two deshielded phenolic hydroxyl groups. The gross structure of **2** was confirmed by the mass spectrum with $[M+H]^+$ at $m/z = 321$, suggesting the molecular formula $C_{16}H_{16}O_7$, in agreement with the NMR spectra. Finally, the structure of **2** was confirmed as methyl ether of fusarubin by comparison with a published NMR data of isolated methyl ether of fusarubin from the fungus *Fusarium solani* [34,35].

The cytotoxic potential of the two pure compounds **1** and **2** were determined by WST-1 assay against human leukemia cells (K-562) using Triton X-100 ($IC_{50}$: 15.1 μg/mL) and $H_2O_2$ ($IC_{50}$: 12.0 μg/mL). The cells seeded in 96-well plates, then incubated for 5 days, were treated with the compound **1** and **2** at various concentrations (0.5-30 μg/mL). The inhibitory process was

**Table 1**
$IC_{50}$ values of compounds 1 and 2 isolated from the extract of *Cladosporium* sp. against K-562 cell line.

| Test Compound | $IC_{50}$ (μg/mL) |
|---|---|
| Triton-X | 15.1 |
| $H_2O_2$ (Positive Control) | 12.0 |
| Anhydrofusarubin (**1**) | 3.97 |
| Methyl ether of fusarubin (**2**) | 3.58 |

**Table 2**
$LC_{50}$ values of crude fungal extract and some column fractions of *Cladosporium* sp.

| Samples | $LC_{50}$ (μg/mL) | Samples | $LC_{50}$ (μg/mL) |
|---|---|---|---|
| VS | 0.29 ± 0.1 | F-5 | 3.34 ± 0.3 |
| CFE | 9.82 ± 2.6 | F-8 | 1.22 ± 0.2 |
| F-1 | 23.58 ± 2.5 | F-10 | 2.09 ± 0.5 |
| F-2 | 42.87 ± 2.3 | F-13 | 1. 4 ± 0.3 |
| F-3 | 22.77 ± 2.9 | F-15 | 0.73 ± 0.2 |
| F-4 | 16.23 ± 1.7 | F-16 | 0.64 ± 0.3 |

Values are expressed as mean ± SEM of three independent experiments. VS: vincristine sulphate used as anticancer standard; CFE: crude fungal extract. F-1, F-2, F-3, F-4, F-5, F-8, F-10 and F-13 are column fractions of fungal extract.

**Table 3**
Antibacterial activity of crude fungal extracts, some column fractions and isolated pure compound **2** of *Cladosporium* sp.

| Microorganism | Zone of inhibition (diameter in mm) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CFE | F-11 | F-12 | F-13 | F-14 | F-15 | F-16 | C-**2** | K |
| Bacteria (Gram +) | | | | | | | | | |
| *S. aureus* | 15 | 14 | 13 | 11 | 17 | 18 | 15 | 27 | 32 |
| *B. megaterium* | 14 | 12 | 16 | 10 | 18 | NA | 31 | 22 | 32 |
| Bacteria (Gram +) | | | | | | | | | |
| *E. coli* | 18 | 10 | NA | 13 | 10 | 17 | 10 | 25 | 30 |
| *P. aeruginosa* | 16 | 10 | 12 | 11 | 13 | 16 | 20 | 24 | 30 |

K: Kanamycin, used as antibacterial standard (30 μg/disc); CFE: Crude Fungal Extract; Column F-11, F-12, F-13, F-14, F-15 and F-16 are column fractions of fungal extract, C-2: Compound 2, purified from F-15 and F-16; NA: no activity.

assessed by using WST-1 (4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2*H*-5-tetrazolio)-1,3-benzene disulfonate) assay [28,29]. Both compounds showed promising inhibition against human leukemia cell (K-562) with the $IC_{50}$ value as 3.97 μg/mL and 3.58 μg/mL, respectively (Table 1).

On the other hand, crude fungal extract and column fractions were tested for cytotoxicity against brine shrimp nauplii. The $LC_{50}$ values (Table 2) were obtained from the best-fit line slope of the curve of% mortality against log values of test sample concentration. Crude fungal extract and column fractions (F-1, F-2, F-3, F-4, F-5, F-8, F-10, F-13, F-15 and F-16) exhibited cytotoxicity with $LC_{50}$ values of 9.82, 23.58, 42.87, 22.77, 16.23, 3.34, 1.22, 2.09, 1.40, 0.73, and 0.64 μg/mL, respectively. By comparing these values with vincristine sulphate as positive control, it was found that crude fungal extract as well as polar column fractions showed potent cytotoxicity to brine shrimp. Among the column fractions, F-8, F-10, F-13, F-15 and F-16 exhibited highest lethality in brine shrimp bioassay (Table 2).

Further, the crude fungal extract (100 μg/disc), column fractions (100 μg/disc), and the isolated compounds 2 (40 μg/disc) were evaluated for antibacterial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus megaterium*. Crude fungal extract, column fractions (F-11, F-12, F-13, F-14, F-15 and F-16) and isolated compound 2 showed moderate to quite promising antibacterial activities (Table 3). Among the column fractions, F-15 and F-16 showed highest activities against gram positive and gram negative bacteria. Other column fractions were also screened but did not show any activities against the tested microorganisms; hence data are not presented in the Table. Fractionation of crude extracts yielded fractions containing different types of compounds based on polarity. Hence, different fractions may possess compounds of different functional groups which may impart different level of antibacterial activities. Comparing with Kanamycin (30 μg/disc), compound 2 exhibited an average zone of inhibition of 27 mm, 25 mm, 24 mm and 22 mm against *S. aureus*, *E. coli*, *P. aeruginosa* and *B. megaterium* respectively. It is to be noted here, compound **2**, identified as methyl ether of fusarubin, was isolated from the bioactive column fractions F-15 and F-16. Our findings indicate that *Cladosporium* sp. lives in *Rauwolfia serpentina* produces useful cytotoxic and pesticidal secondary metabolites. It is in line with the recent reports on endophytic fungi having biological activities, particularly producing cytotoxic metabolites such as norsesquiterpenes, sesquiterpenes, tetracyclic polyketide etc [21,36–38].

## 4. Conclusion

In conclusion, the present works have provided two antibacterial and cytotoxic secondary metabolites from the fungal strain *Cladosporium* sp. which were established as anhydrofusarubin (**1**) and methyl ether of fusarubin (**2**). This is the first time report of compounds **1** and **2** from the endophytic fungus *Cladosporium* species. The study has also revealed that the *Cladosporium* species obtained from *Rauwolfia serpentina* contains potent cytotoxic, antibacterial and antifungal metabolites. This discovery indicated that endophytic fungi of *Rauwolfia serpentina* have significant scientific and industrial potentials, and could be an ideal source for the discovery of potential bioactive compounds or leads for the future drugs.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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