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ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI ISOLATED FROM *Ophiorrhiza baviensis* AS A SOURCE OF POTENTIAL ANTICANCER AND ANTIOXIDANT COMPOUNDS

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ABSTRACT

Endophytic fungi present in internal tissues of medicinal plants are a potent source of natural products with pharmacological and medical activities. Hence, the present study aimed to isolate endophytic fungi from *Ophiorrhiza baviensis* Drake and assess their anticancer and antioxidant properties. Twelve fungal endophytes were recovered from *O. baviensis* growing in Cuc Phuong National Park, in which fungi associated with leaves occupied predominantly. Using morphological and ITS sequence analyses, fungal strains belonged to Ascomycota, with three different genera *Colletotrichum* (8 strains), *Penicillium* (2 strains), and *Diaporthe* (2 strains). The anticancer assays of cancer cell A549 were carried out for the dichloromethane-methanol extracts of these strains. The crude extract of *Colletotrichum kahawae* XCB2.L7 showed the highest anticancer activities against A549 at the concentration of 20 µg/mL. The TLC analysis revealed that the anticancer drug camptothecin was likely accumulated in mycelia. Further evaluation of anticancer activities revealed the IC_{50} values of DPPH, hydroxyl radicals, and superoxide radicals were determined to be 431.5 ± 12.4 µg/mL, 534.24 ± 8.4 µg/mL, and 487.0 ± 9.2 µg/mL, respectively. In line with antioxidant activity, *C. kahawae* XCB2.L7 produced high levels of polyphenol (89.1 ± 5.4 µg GAE/g FW) and flavonoid (114.2 ± 8.6 µg GAE/g FW) contents. These findings shed new light on endophytic fungi from *O. baviensis* and provided a promising source for exploration of new and commercial compounds.

Keywords: Anticancer, antioxidant, camptothecin, endophytic fungi, *Ophiorrhiza baviensis*

INTRODUCTION

Cancer remains a major cause of death and morbidity in the world, with an estimated 19.3 million new cancer cases and 10 million cancer deaths in 2020 (Sung *et al.*, 2021). Chemotherapy based on the drugs is an effective approach to treat cancer cells. However, the effectiveness is significantly decreased due to the development of drug

resistance in cancer cells (Perillo *et al.*, 2020). Taking into account, new drugs used for chemotherapy treatments more effective for patients are urgently needed. Apart from that, an imbalance of reactive oxygen species (ROS) and antioxidants in the human body is named oxidative stress, which results in severe damage of macromolecules such as DNA, RNA, lipids, and protein (Tung *et al.*, 2020). As a result, the accumulation of ROS-induced damage can contribute to genetic instability and the emergence of cancer cells (Perillo *et al.*, 2020). On this account, the discovery of novel natural products with unique chemical structures and versatile biological activities from untapped or unique ecosystems has been paid attention to date.

Fungal endophytes play an important role in plant protection against pathogens, adaptation to adverse environments, and plant-growth promotion (Grabka *et al.*, 2022). It is attributed to the ability to produce many biologically active compounds with excellent bioactivities such as antibacterials, antifungals, antivirals, and anticancer (Hashem *et al.*, 2023). *Fusarium foetens* AQF6 was isolated from *Amentotaxus yunnanensis*, where fusafoetriol showed strong antioxidant activity (Vu *et al.*, 2024). Tumor cell lines such as A549, LN229, MGC, LOVO, and MDA231 were inhibited by 3 α-pyrone derivatives extracted from the endophytic fungus *Penicillium ochrochloronthe* (Zhao *et al.*, 2019). Of note, endophytic fungi are able to mimic plant metabolites because they are likely to integrate the metabolic pathways of host plants and obtain genetic materials (Hashem *et al.*, 2023). For example, our earlier study showed that the anticancer drug camptothecin is produced by *Penicillium* sp. I3R2 recovered from *Ixora chinensis* in Vietnam (Doan *et al.*, 2024). Likewise, *Fusarium solani* PQF9 was isolated from *Podocarpus pilgeri* and showed anticancer activity where the most wanted anticancer drug, paclitaxel, was separated (Vu *et al.*, 2023).

It is well-documented that fungi from medicinal plants are considered as a treasure house for developing anticancer and antioxidant drugs. However, there is no report concerning the anticancer and antioxidant activities of endophytes from *O. baviensis* Drake growing in Vietnam. Known as a flowering plant in the Rubiaceae family, *O. baviensis* has similar morphology to *Ophiorrhiza liuyanii* and is distributed in southwestern China and northern Vietnam (Pham *et al.*, 2023). This study aimed to isolate and evaluate the anticancer and antioxidant properties of endophytic fungi isolated from the *O. baviensis* collected in Cuc Phuong National Park, Vietnam.

MATERIALS AND METHODS

Fungal isolation

O. baviensis Drake, divided into 3 parts such as roots, stems and leaves, was sampled at Cuc Phuong National Park, Vietnam, in 2020. The voucher specimens were classified and then deposited in the herbarium of the Institute of Ecology and Biological Resources, Hanoi, under accession number: Xacan 01.

For fungal isolation, the surface sterilization method was used as previously described (Doan *et al.*, 2024). In brief, each plant sample was first washed 3 times under running tap water and then 2 times with deionized water to remove soil particles and epiphytic microbiota. Samples were dried by putting them on sterile papers under aseptic conditions, which were subsequently treated

with 75% (v/v) ethanol for 3 min and NaOCl for 3 min. After washing with deionized water 3 times, all samples were dried with sterile filter papers in order to cut into small segments. In the final step, samples were aseptically put on the interior surface of Potato Dextrose Agar (PDA) plates supplemented with 0.5 g/L penicillin and 1 g/L streptomycin. After 15 days of incubation at 28°C in the dark, fungal colonies grown on the plant tissues were selected and streaked out on the new PDA plates. Once fungal isolates were pure, 15% (v/v) glycerol was prepared to store all strains and then used for further experiments.

Identification of fungal endophytes

The growth, morphology of colonies, and pigment production were observed on the PDA plates at 28°C for 5 days. In addition, hyphae and spores were determined through a light microscope at 40X.

The genomic DNA was extracted using an AllPrep Fungal DNA/RNA/Protein Kit (Qiagen) and the quality of genomic DNA was observed on a 0.8% agarose gel. The fungal internal transcribed spacer (ITS) gene was amplified using ITS1 and ITS4 primers. After PCR amplification described previously (Quach Ngo *et al.*, 2022), the amplicons were checked by agarose gel electrophoresis followed by PCR purification steps. Sequence data was obtained by the ABI Prism 3500 XL sequencer (Applied Biosystems) at the National Key Laboratory of Gene Technology, Institute of Biotechnology. The ITS sequences were compared to sequences found in the GenBank database (NCBI) and then used to build the phylogenetic tree using Molecular Evolutionary Genetics Analysis v.11.0.

Anticancer assay

Fermentation was carried out in 1 L flasks containing 300 mL of Potato Dextrose Broth (PDB) at 28°C for 8 days. After that, it was ended with the addition of 900 mL solvent mixture containing dichloromethane and methanol (9:1, v/v). The flasks were shaken at 100 rpm for 16 hours to allow complete extraction. The extracts were concentrated to dryness by a vacuum rotary evaporator and kept at 4°C for further experiments.

The cytotoxicity of the fungal extracts on human lung cancer A549 cells was performed using the sulforhodamine B (SRB) method as previously described (Vu *et al.*, 2023). Cells were seeded in 96-well plates with a density of 6×10^3 cells/well at 37°C and 5% CO_2. Stock solutions of the fungal extracts were prepared in 10% DMSO and then added to the 96-well plate to reach a final concentration of 20 μg and 100 μg. About 20% (w/v) trichloroacetic acid (TCA) was added to each well in order to mix 0.4% (w/v) SRB solution for 30 min at 37°C. The plates were gently shaken for 5 min and de-stained by 1% (v/v) acetic acid for 3 times. The optical density was measured using the ELISA Plate Reader (Biotek) at 540 nm.

Antioxidant activity

The antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was performed following the previous procedure (Quach Vu *et al.*, 2022). About 0.1 mL of 0.1 mM DPPH solution in ethanol was reacted with 0.1 mL of different doses of fungal extract (0.01, 0.02, 0.04, and 0.06 mg/mL) at room temperature for 30 min. The optical density was measured at 517 nm against an equal amount of DPPH using a microplate reader. The ability to scavenge

hydroxyl radicals of the fungal extract was evaluated as described previously (Vu *et al.*, 2022). The mixture consisting of 0.05 mL of 0.435 mM brilliant green, 0.1 mL of 0.5 mM $FeSO_4$, and 0.075 mL of 3% (v/v) H_2O_2 was prepared. Then 0.1 mL of different doses of fungal extract (0.01, 0.02, 0.04, and 0.06 mg/mL) was subjected to the mixture at room temperature for 30 min. Absorbance at 624 nm was utilized.

Determination of total polyphenol and flavonoid contents

The Folin-Ciocalteu colorimetric method was used to determine total phenolic content (da Silva *et al.*, 2020). The fungal extract solution was prepared in 70% ethanol and then mixed with 100 µL of Folin-Ciocalteu reagent. After 5 min, 80 µL of 4% (w/v) sodium carbonate was added to terminate the reaction and absorbance at 765 nm was measured by using a microplate reader. The gallic acid equivalents (GAE) in µg per g of fungal extract (µg GAE/g FW) were calculated based on the gallic acid standard curve. The total flavonoid content was obtained according to the $NaNO_2$-$Al(NO_3)_3$ colorimetry assay (Tang *et al.*, 2020). About 30 µL of 70% ethanol extract was subjected to the mixture containing 10 µL of 5% (w/v) $NaNO_2$, 10 µL of 10% (w/v) $AlCl_3$, 60 µL of 1M NaOH, and 120 µL of distilled water for 30 min at room temperature. The measurement was performed at 510 nm in order to calculate quercetin equivalents in microgram per gram (µg QE/g) of dry extract (µg QE/g FW).

RESULTS AND DISCUSSIONS

Isolation and distribution of endophytic fungi from *O. baviensis*

In this experiment, no fungal colony was grown on the PDA plates, indicating the effectiveness of the sterilization method. After purification, a total of 12 fungal isolates was successfully isolated from *O. baviensis*. Among them, 6 strains (50%) were recovered from leaves, 4 strains (33%) originated from stems, and 2 strains (17%) were purified from roots (Figure 1). It indicated that *O. baviensis* could be a suitable host for the endophytic fungi. The present study was similar to the report of (Ahmad *et al.*, 2022) on fungal endophytes of *Acacia mangium*. It could be that environmental fungi enter plant tissue through water holes, wounds, etc., to be endophytic, which results in high abundance. In contrast, the stem part of *Cotoneaster multiflorus* showed the highest number of fungi, followed by roots and leaves (Lü *et al.*, 2023).

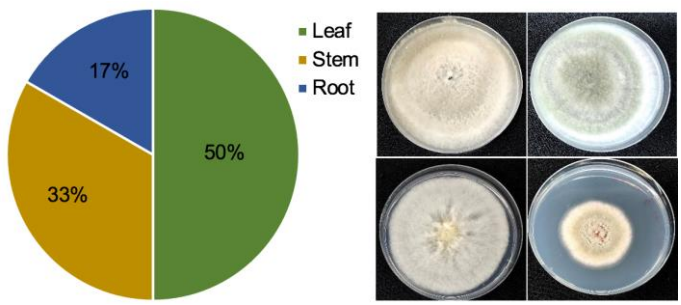

Figure 1. Distribution and morphology of endophytic fungi isolated from *O. baviensis*

Identification of endophytic fungi from *O. baviensis*

Morphological analysis revealed that fungal endophytes grew well on the PDA plates and

showed mainly the color from white to gray (Figure 1). Eight isolates, including XCB2.L3, XCB3.L4, XCC2.T4, XCB2.L7, XCC2.T10, XCB1.L12, XCA1.T2, and XCC3.T8, were supposed to be members of *Colletotrichum*, while XCA1.R2 and XCA3.R2 were grouped into *Penicillium*. In addition, XCB1.L17 and XCB3.L2 belonged to *Diaporthe*.

With the aim to identify 12 fungal isolates at the species level, ITS gene sequence analysis was carried out. It turned out that the ITS sequences of fungal isolates showed 96.13–98.82% identity with those of the related species. Phylogenetic analysis of ITS sequences of fungal strains revealed that 8, 2, and 2 strains were clustered into *Colletotrichum*, *Penicillium*, and *Diaporthe* genera, respectively (Figure 2). These results were in agreement with morphological results. Therefore, fungal isolates were identified as *Colletotrichum boninense*.

(XCB2.L3, XCB1.L12), *Colletotrichum fici* (XCB3.L4), *Colletotrichum tropicicola* (XCC2.T4), *Colletotrichum kahawae* (XCB2.L7), *Colletotrichum fioriniae* (XCC2.T10), *Colletotrichum ti* (XCA1.T2), *Colletotrichum cigarro* (XCC3.T8), *Fusarium longifundum* (XCA1.R2), *Fusarium ambrosium* (XCA3.R2), *Diaporthe sackstonii* (XCB1.L17), and *Diaporthe bounty* (XCB3.L2).

Since *Ophiorrhiza mungos*, *O. pumila*, *O. liukiuensis*, *O. trichocarpon*, and *O. pectinata* are known to produce camptothecin (Rajan *et al.*, 2013; Yang *et al.*, 2021), fungal endophytes have been investigated. For example, 16 fungal strains were isolated from *O. mungos*, however, only camptothecin-producing fungi were identified (Aswani *et al.*, 2020). To the best of our knowledge, this study for the first time shed light on culturable fungi colonizing on *O. baviensis*.

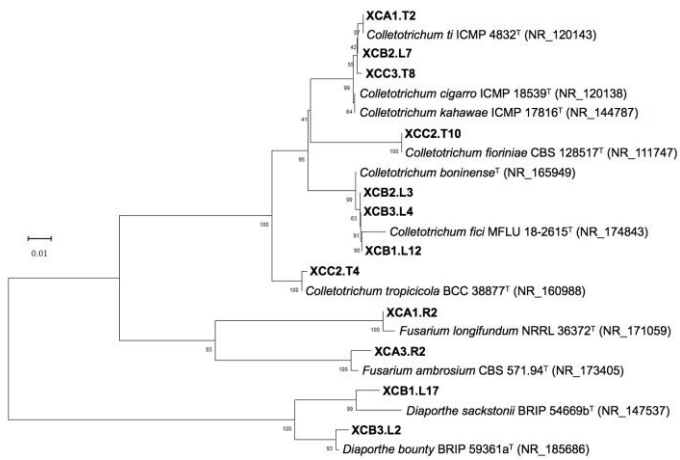

Figure 2. Phylogenetic analysis based on the ITS sequences of 12 fungal strains from *O. baviensis* with reference ITS sequences

Anticancer screening of the crude extracts from endophytic fungi cultures

All fungal cultures were fermented and extracted to obtain the crude extracts to evaluate cytotoxic activity against cancer cell A549. It revealed that 12 fungal extracts showed an inhibitory effect on A549 cells with different levels. At 100 µg extract, 6 extracts of endophytic fungi showed significant inhibitory activities against A549

with the percent inhibition ranging from 82.33 ± 1.19% to 96.47 ± 2.25% (Table 1). Using 20 µg extract, only *C. kahawae* XCB2.L7 demonstrated the highest anticancer activity with a percent inhibition of 40.25 ± 0.39%. Based on the recommendation from the National Cancer Institute, extracts with $IC_{50} \leq 20$ µg/mL are highly cytotoxic and IC_{50} ranging between 21 and 200 µg/mL is moderately cytotoxic (Nguyen *et al.*, 2020). Therefore, the XCB2.L7 extract indicates good cytotoxicity.

Table 1. Cytotoxic activity of endophytic fungi fermented extracts

Endophyte code	Closest match	Percent inhibition against A549 cell line (%)	
		20 µg	100 µg
XCB2.L3	*Colletotrichum boninense*	16.04 ± 0.19	78.39 ± 1.47
XCB3.L4	*Colletotrichum fici*	16.53 ± 1.29	44.06 ± 1.24
XCA1.R2	*Fusarium longifundum*	6.82 ± 0.29	46.80 ± 2.06
XCC2.T4	*Colletotrichum tropicicola*	16.49 ± 1.76	82.33 ± 1.19
XCB1.L17	*Diaporthe sackstonii*	35.29 ± 0.94	82.64 ± 1.42
XCB3.L2	*Diaporthe bounty*	16.82 ± 0.43	95.45 ± 0.86
XCB2.L7	*Colletotrichum kahawae*	40.25 ± 0.39	79.10 ± 0.94
XCC2.T10	*Colletotrichum fioriniae*	12.65 ± 0.50	73.47 ± 2.04
XCB1.L12	*Colletotrichum boninense*	18.18 ± 1.28	96.47 ± 2.25
XCA1.T2	*Colletotrichum ti*	22.51 ± 1.18	82.84 ± 1.77
XCC3.T8	*Colletotrichum cigarro*	87.92 ± 1.92	87.92 ± 1.92
XCA3.R2	*Fusarium ambrosium*	26.54 ± 1.71	26.54 ± 1.71

C. kahawae is an important phytopathogenic fungus causing coffee berry disease all over the world (Vieira *et al.*, 2019). It has been reported that *Colletotrichum* species can be plant pathogens, saprobes, and endophytes, which supported that *C. kahawae* XCB2.L7 could be an endophyte. Importantly, the anticancer activity of *C. kahawae* has not been reported yet. Therefore, purification and structural elucidation will be interesting subjects for future studies.

Potential of producing camptothecin

The cell-free supernatant and mycelia of *C. kahawae* XCB2.L7 were extracted with dichloromethane and methanol to yield brown residues. The TLC analysis revealed that the cell extract had the spot identical to the standard camptothecin band but not with the supernatant crude extract (Figure 3). Both standard camptothecin and XCB2.L7 extract had the Rf values of 0.5. *Colletotrichum fructicola* isolated from *Nothapodytes nimmoniana* was reported to produce camptothecin (Tiwari & Bae, 2022). To date, no report has demonstrated the ability to produce camptothecin of *C. kahawae*. Future studies are required to confirm whether camptothecin is produced by *C. kahawae* XCB2.L7.

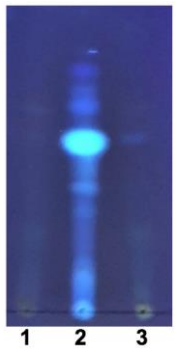

Figure 3. TLC of supernatant crude (1) and cell extracts (3) along with standard camptothecin (2)

Antioxidant activity

It is believed that free radicals causing oxidative stress result in the emergence of cancers (Tung *et al.*, 2020). The antioxidant activities of the XCB2.L7 extract were tested against free radicals such as scavenging hydroxyl, DPPH, and superoxide anion radicals. The IC_{50} value of DPPH for the XCB2.L7 extract was found to be 431.5 ± 12.4 µg/mL (Figure 4A). It indicated that the XCB2.L7 extract had the ability to eliminate *DPPH* radicals by donating electrons, which was represented by a color shift in the assay. In addition, the XCB2.L7 extract also displayed moderate antioxidant activity against hydroxyl radicals and superoxide radicals with an IC_{50} value of 534.24 ± 8.4 µg/mL and 487.0 ± 9.2 µg/mL, respectively. This result was consistent with a previous study where the plant *Ginkgo biloba* L. is a host of *Aspergillus nidulans* ST22 and *Aspergillus oryzae* SX10 capable of producing strong antioxidant activities (Qiu *et al.*, 2010). Of note, the IC_{50} values for 3 oxidant radicals were comparable to those of endophytic fungi isolated from *Cordia dichotoma* (Sharma *et al.*, 2023). The antioxidant scavenging activities of the XCB2.L7 extract could be due to its hydrogen donating ability and superoxide dismutase-like properties to scavenge free radicals. Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate are previously used, however, side effects have been reported to be serious issues (Turkoglu *et al.*, 2007).

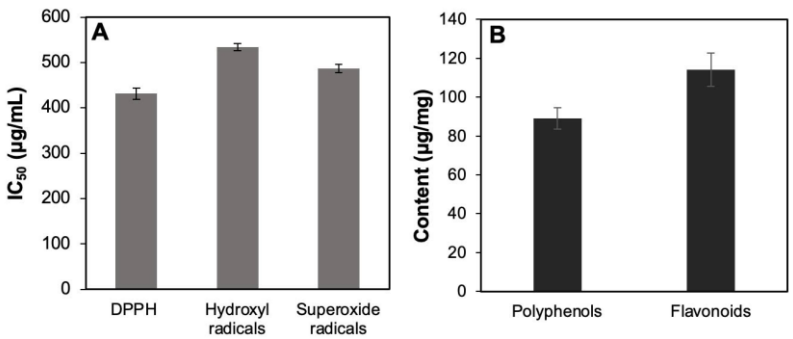

Figure 4. Antioxidant activity (A) and phytochemical concentrations (B) determined in the crude extract of *C. kahawae* XCB2.L7

In line with antioxidant activity, *C. kahawae* XCB2.L7 also produced both polyphenol (89.1 ± 5.4 µg GAE/g FW) and flavonoid (114.2 ± 8.6 µg GAE/g FW) contents (Figure 4B). Phytochemical levels of the XCB2.L7 were higher than those of endophytic fungi from *Tsuga chinensis* growing in Vietnam (Vu *et al.*, 2022). The crude extract of *Penicillium oxalicum* YMG1 isolated from *Ligusticum chuanxiong* Hort was found to contain polyphenol compounds including hesperidin, citric acid, ferulic acid, and alternariol, which were responsible for strong antioxidant activities against DPPH, superoxide, and hydroxyl radicals (Tang *et al.*, 2020). A linear correlation between high phytochemical content and antioxidant activity was reported in *Cladosporium cladosporioides* (Sharma *et al.*, 2023). In contrast, a total phenolic content of *Aspergillus brasiliensis* MCR1 was not related to the antioxidant activity (Sharma *et al.*, 2023), suggesting the presence of other antioxidant compounds. Therefore, the endophytic fungus *C. kahawae* XCB2.L7 may be a producer of antioxidant compounds against oxidative damage.

CONCLUSION

Endophytic fungi capable of producing pharmacological and medicinal compounds are known to relate to medicinal plants. For the first time, this study shed light on the endophytic fungi from *O. baviensis* collected in Cuc Phuong National Park. Among 12 culturable fungal strains, the endophytic fungus *C. kahawae* XCB2.L7 exhibited excellent anticancer and antioxidant activities. In addition, this strain also potentially produced camptothecin. This study provided the potent candidate for the discovery of new bioactive secondary metabolites as well as camptothecin. The combination of chemical analysis and whole genome sequencing is needed for further studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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