บทความวิจัย

สมบัติการต้านอนุมูลอิสระและความเป็นพิษต่อเซลล์มะเร็งของเชื้อราเอนโดไฟต์จากชาน้ำมันสกุล *Camellia oleifera* Abel

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บทคัดย่อ

เชื้อราเอนโดไฟต์ 17 ชนิดได้ถูกค้นพบจากส่วนต่างๆ ของต้นชาน้ำมันสกุล *Camellia oleifera* Abel และเมื่อนำมาทำการศึกษาฤทธิ์ต้านอนุมูลอิสระและฤทธิ์เป็นพิษของสารสกัดหยาบจากน้ำเลี้ยงเชื้อ (broth crude extracts) ราเอนโดไฟต์หมายเลข L004 แสดงฤทธิ์ต้านอนุมูลอิสระและเป็นพิษต่อเซลล์มะเร็งได้ และสารสกัดดังกล่าวมีฤทธิ์ต้านอนุมูลอิสระมากกว่า 62% เมื่อทำการทดสอบด้วยวิธี DPPH และ xanthine / xanthine oxidase (XXO) นอกจากนี้ยังแสดงการยับยั้งการต้านอนุมูลอิสระแบบ superoxide anion ที่เกิดจากการกระตุ้นด้วย TPA ในเซลล์มะเร็งเม็ดเลือดขาวชนิด HL-60 มากกว่า 62% และมีผลการต้านอนุมูลอิสระในหน่วยของ ORAC สูงกว่า 2.0 อีกทั้งสารสกัดหยาบของเชื้อราเอนโดไฟต์หมายเลข L004 ยังแสดงความเป็นพิษต่อเซลล์มะเร็ง โดยสามารถยับยั้งเซลล์มะเร็งเม็ดเลือดขาวชนิด Molt-3 ได้ถึง 84% ทั้งนี้ยังมีสารสกัดหยาบของเชื้อราเอนโดไฟต์หมายเลขอื่นๆ ที่มีความเป็นพิษต่อเซลล์มะเร็งได้ อาทิ เชื้อราเอนโดไฟต์หมายเลข L005 T001 และ T002 มีความเป็นพิษต่อเซลล์ Molt-3 ได้มากกว่า 57% และเชื้อราเอนโดไฟต์หมายเลข F001 มีความเป็นพิษต่อทั้งเซลล์ Molt-3 และเซลล์มะเร็งตับชนิด HepG2 ที่ 88% และ 55.73% ตามลำดับ ผลการทดลองนี้แสดงให้เห็นว่าเชื้อราเอนโดไฟต์จากส่วนต่างๆ ของต้นชาน้ำมันสกุล *Camellia oleifera* Abel นั้นอาจเป็นแหล่งที่มีศักยภาพในการหาสารที่มีคุณสมบัติในการต้านอนุมูลอิสระและสารต้านเซลล์มะเร็งได้

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Antioxidant and Cytotoxic Activities of Endophytic Fungi from *Camellia oleifera* Abel

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ABSTRACT

Seventeen endophytic fungi were isolated from several parts of *Camellia oleifera* Abel and the antioxidant and cytotoxic activities of the broth crude extracts were determined. The broth crude extract of endophytic fungi No. L004 exhibited promising antioxidant and cytotoxic activities. It showed high antioxidant activity in both DPPH and xanthine/xanthine oxidase (XXO) assay, higher than 62% inhibition. It also displayed inhibition of TPA-induced superoxide anion radical formation in differentiated HL-60 human promyelocytic leukemia cell lines, at 62% inhibition, and revealed high ORAC antioxidant activity with 2.0 ORAC unit. Moreover, the endophytic fungus No. L004 showed good cytotoxic activity in Molt-3 (acute lymphoblastic leukemia) cell lines at 84% inhibition. Other endophytic fungi (No. L005, T001, and T002) also displayed the cytotoxic activity on Molt-3 cell lines, higher than 57% inhibition. The endophytic fungus No. F001 inhibits both Molt-3 and HepG2 (human hepatocellular liver carcinoma) cell lines at 88% and 55.73% inhibition, respectively. These results indicate that endophytic fungi from *Camellia oleifera* Abel could be a promising source for antioxidant and anticancer agents.

Keywords: *Camellia oleifera* Abel, endophytic fungi, antioxidant, cytotoxicity

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Introduction

Endophytic fungi are fungi isolated from plants. There are many varieties of these endophytic fungi around the world [1]. Endophytic fungi produces diverse structural secondary metabolites such as terpenoids, furandiones, palmarumycins, dimeric anthrone, phenols, and benzopyroanone [2]. The biological activities of secondary metabolites from endophytic fungi have been studied extensively and results showed that endophytic fungi are a rich source of bioactive compounds which can be used as potential leads for novel medicines [1-3]. Several important pharmaceutical compounds were derived from endophytic fungi [4], for example, podophyllotoxin [5], paclitaxel (Taxol) [6], hypericin [7], and camptothecin [8].

Camellia oleifera Abel known as tea oil, is a shrub tree with a height of 15-20 feet. It belongs to the Camellia family that originates from China and can also be found in the northern region of South East Asia. It has a white color flowering which normally blooms in winter. The fruit is a small round shape with a length of 0.5-1 inch and covered with a dry and hard shell [9]. The seeds are commonly used to make cooking oil, which contains several chemical constituents such as fatty acids [10] and 2,5-bis-benzo(1,3)dioxol-5-yl-tetrahydro-furo (3,4-d)(1,3)dioxine [11]. The later compound showed good antioxidant properties. In recent years, the biological activities of the chemical compounds isolated from different parts of *C. oleifera* were studied. Polysaccharides from the seed cake and fruit shell shows antioxidant and antitumor activities [12-13]. Biflavonoid from the fruit shells showed free radical scavenging activities in both *in vitro* and *in vivo* assays [14]. Sasanquasaponin from the seed cake displayed anticancer activity by inducing cell cycle arrest and apoptosis in MCF-7 human breast cancer cells [15]. However, there is no information available about the studies of endophytic fungi from *C. oleifera.*

In this study, endophytic fungi from several parts of *C. oleifera* was isolated and extracted. Here in, we reported the first findings of the antioxidant and cytotoxicity activities of these fungal extract. Results from this study would provide valuable information for the development of novel medicinal agents.

Materials and Methods

Plant materials

Bark, leaves, twigs, and fruits of *C. oleifera* were collected at November 2016 from the Tea Oil and Plant Oils Development Center, Mae Sai, Chiang Rai, Thailand.

Isolation of endophytic fungi

Plant samples were cleaned under running tap water and then air-dried. Before surface sterilization, the cleaned stems (bark, leaves, twigs and fruits) were cut into small pieces of ca. 5 cm long leaves. The fragments were sterilized in 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min, and sterile distilled water for 1 min two times and air-dried in a laminar flow chamber. The surface-sterilized leaves and stems were cut into small pieces using a sterile blade and transfer to sterile water agar plates for incubation at 30°C. The hyphal tip of endophytic fungi growing out from the plant tissue was cut by a sterile pasture pipette and transferred to a sterile potato dextrose agar (PDA) plate. After incubation at 30°C for 7-14 days, pure culture was determined from colony morphology [16-17]. All of the endophytic fungi cultures were deposited at Center of Excellence in Fungal Research Mae Fah Luang University.

Fermentation and extraction

Endophytic fungi isolates were grown on potato dextrose agar (PDA) plate at 30°C for 7 days. Six pieces (6×6 mm^2) of the grown culture cut from the plate were incubated into a 1000 ml Erlenmeyer flask containing 250 mL of potato dextrose broth (PDB). After incubation at 25°C for 21 days under stationary condition, the fungal culture was filtered to remove the mycelium. The filtrate broth crude was partitioned with an equal volume of ethyl acetate for three times to obtain the broth extract.

Cytotoxic assay

HepG2 (human hepatocellular liver carcinoma), HuCCA-1 (human lung cholangiocarcinoma), and A549 (human lung carcinoma) cancer cell lines were evaluated the cytotoxic activity with broth crude extracts by using MTT assay [18], while Molt-3 (acute lymphoblastic leukemia) and HL-60 (human promyelocytic leukemia) cell lines were investigated with broth crude extracts by using XTT assay [19]. The reference drugs were doxorubicin hydrochloride and etoposide.

Scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals

The activity on radical scavenging was evaluated. The solvent was used as a blank (0% radical scavenging) and 250 μM at final concentration of ascorbic acid as the reference compound (100% radical scavenging). The half maximal scavenging concentration or IC_{50} value of ascorbic acid is at 21.2 μM. This assay was carried out as described by Gerhäuser et al [20].

Inhibition of superoxide anion radical formation by xanthine/xanthine oxidase (XXO assay)

This assay was performed following the described method by Gerhäuser et al [20]. 30U of superoxide dismutase enzymes was used as a negative control, which has 100% radical scavenging. The IC_{50} value of the tested compound represents the half of the maximal scavenging concentration (50% inhibition of the radicals formed). Allopurinol was used as the reference compound which showed the inhibition on xanthine oxidase (IXO) at IC_{50} value of 3.0 μM. Inhibition of superoxide anion radical formation was measured only when the tested compounds did not inhibit xanthine oxidase.

Inhibition of 12-O-tetra-decanoylphorbol-13-acetate (TPA)-induced superoxide anion radical generation in differentiated HL-60 cell lines (HL-60 assay)

TPA-induced superoxide anion radical formation in differentiated HL-60 (human promyelocytic leukemia) cells was detected by photometric determination of cytochrome c reduction. This assay was carried out according to the method described by Gerhäuser and coworker [20] 12U of superoxide dismutase was used as a positive control which shows as 100% radical scavenging. The concentration of 50% inhibition that exhibits superoxide anion radical formation is the IC_{50} value. This assay considered only the sample that has more than 50% cell viability for the calculation of scavenging potential.

Measurement of oxygen radical absorbance capacity (ORAC)

ORAC assay measures the peroxyl radical absorbance capacity of compounds which was described by Gerhäuser et al [20]. Antioxidant potential of test compounds (1 μM) was compared with the potential of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The results were shown as ORAC unit which 1 ORAC unit equals the net protection of β-phycoerythrin produced by 1 μM Trolox. The positive data are the scavenging capacities more than 1 ORAC unit.

Inhibition of aromatase (CYP19)

Aromatase inhibition assay was carried out according to the method described by Stresser et al [21]. Ketoconazole was used as a positive control with IC_{50} value of 2.4 μM.

Results

Isolation of endophytic fungi

Seventeen endophytic fungi were isolated from *C. oleifera.* The results of the morphology of the endophytic fungi from various planting material were contributed as shown in Figure 1. All of the cultures from the endophytic fungi were deposited at the Center of Excellence in Fungal Research-Mae Fah Luang University to generate MFLUCC codes and identified the genus (Table1).

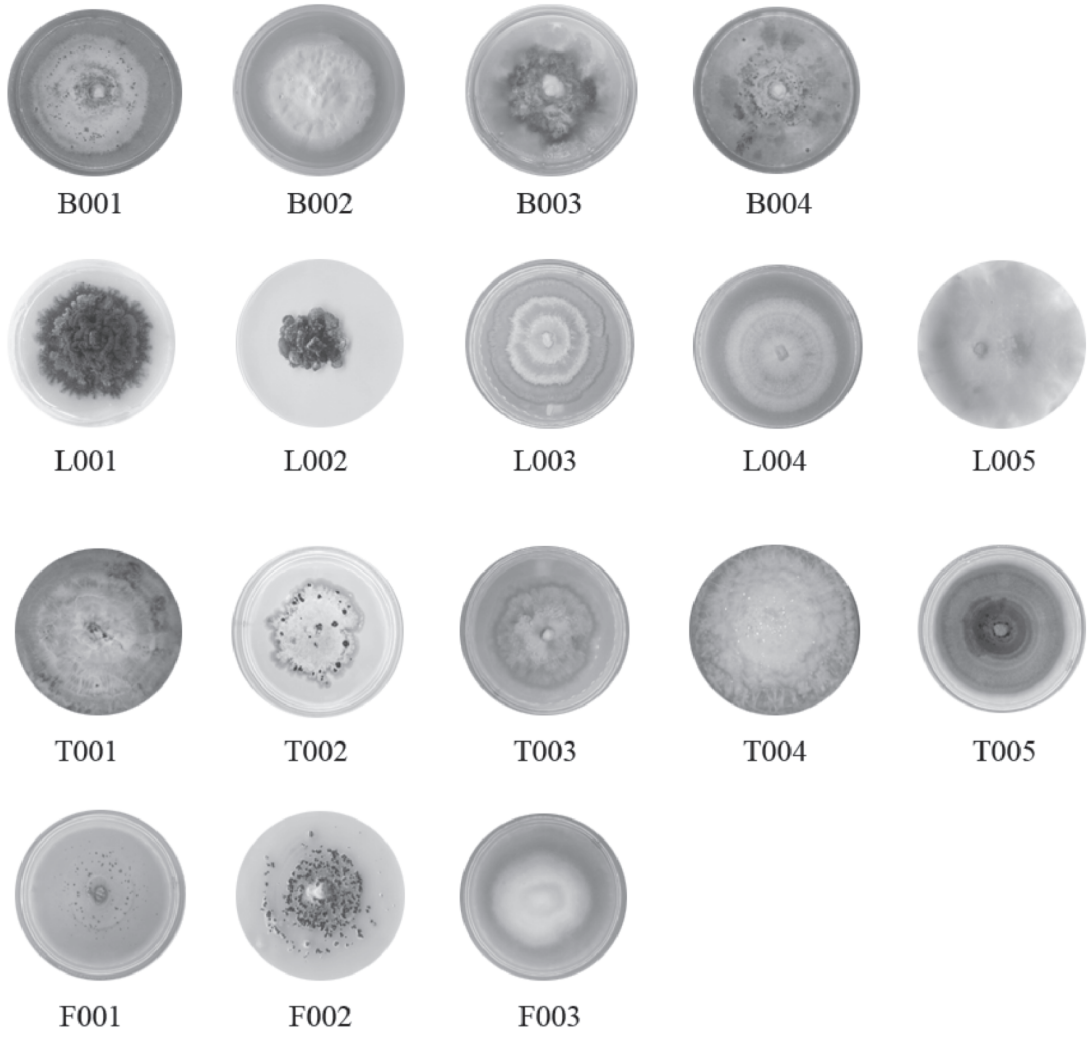

Figure 1 The pictures of seventeen endophytic fungi isolated from *Camellia oleifera* Abel. (Plant part abbreviation: B-bark, L-leaves, T-twig, F-fruit)

Table 1 The list of isolated endophytic fungi from Camellia oleifera Abel.

No.	Original Code	Genus	MFLUCC Code
1	B001	Unknown	MFLUCC15-1139
2	B002	*Pestalotiopsis* sp.	MFLUCC15-1140
3	B003	*Pestalotiopsis* sp.	MFLUCC15-1141
4	B004	*Pestalotiopsis* sp.	MFLUCC15-1142
5	L001	Unknown	MFLUCC15-1143
6	L002	Unknown	MFLUCC15-1144
7	L003	*Pestalotiopsis* sp.	MFLUCC15-1145
8	L004	Unknown	MFLUCC15-1146
9	L005	Unknown	MFLUCC15-1147
10	T001	Unknown	MFLUCC15-1148
11	T002	*Xylaria* sp.	MFLUCC15-1149
12	T003	Unknown	MFLUCC15-1150
13	T004	Unknown	MFLUCC15-1151
14	T005	*Collectotigum* sp.	MFLUCC15-1264
15	F001	Unknown	MFLUCC15-1152
16	F002	Unknown	MFLUCC15-1153
17	F003	*Xylaria* sp.	MFLUCC15-1154

Cytotoxic assay

The cytotoxicity of the broth extracts of the endophytic fungi was evaluated. HepG2, HuCCA-1, A549, and Molt-3 cell lines were used in the cytotoxicity assay and the broth extracts were used at a concentration of 30 μg/mL. Results are shown in Table 2. MTT assay was used to evaluate the cytotoxicity on HepG2, HuCCA-1, and A549 cell lines [19]. In addition, Molt-3 cell lines were studied by using XTT assay [19]. Etoposide and doxorubicin hydrochloride are used as positive controls.

Table 2 Percent inhibitions on cytotoxicity activities of the broth extracts of the endophytic fungi from *Camellia oleifera* Abel at 30 μg/mL by using MTT (HepG2, HuCCA-1, and A549) and XTT (Molt-3) assays.

Original Code	MFLUCC Code	HepG2	HuCCA-1	A549	Molt-3
B001	MFLUCC15-1139	0.17	0.00	20	34
B002	MFLUCC15-1140	-[a]	-[a]	-[a]	-[a]
B003	MFLUCC15-1141	2.21	0.00	7	15
B004	MFLUCC15-1142	0.00	0.00	1	19
L001	MFLUCC15-1143	0.00	0.00	4	1
L002	MFLUCC15-1144	0.00	0.00	9	3
L003	MFLUCC15-1145	0.00	0.00	14	30
L004	MFLUCC15-1146	0.21	0.00	11	84
L005	MFLUCC15-1147	3.80	0.00	13	73
T001	MFLUCC15-1148	0.09	1	22	68
T002	MFLUCC15-1149	33.87	42	30	57
T003	MFLUCC15-1150	9.20	1	0.00	13
T004	MFLUCC15-1151	-[a]	-[a]	-[a]	-[a]
T005	MFLUCC15-1264	-[a]	-[a]	-[a]	-[a]
F001	MFLUCC15-1152	55.73	0.00	20	88
F002	MFLUCC15-1153	-[a]	-[a]	-[a]	-[a]
F003	MFLUCC15-1154	-[a]	-[a]	-[a]	-[a]
Etoposide (μg/mL)		25.05 ± 3.06	-[a]	-[a]	0.041 ± 0.004
Doxorubicin Hydrochloride (μg/mL)		0.30 ± 0.02	0.43 ± 0.08	0.25 ± 0.06	-[a]

[a]Not determined

Antioxidant assay

Antioxidant or potential cancer chemopreventive properties of the broth extracts of the endophytic fungi from *C. oleifera* were evaluated by measuring the radical scavenging, antioxidant activity, and aromatase (CYP19) inhibition. The broth extracts were used at a concentration of 6.25 μg/mL. (Table 3) [10, 20, 22].

Table 3 Radical scavenging, antioxidant, and aromatase inhibitory activities of the broth extracts of endophytic fungi from *Camellia oleifera* Abel at 6.25 μg/mL.

		Radical scavenging and antioxidant activities					
Original Code	**MFLUCC Code**	**DPPH assaya**	**XXOb**	**IXOc**	**HL-60b**	**ORAC (unit)d**	**Inhibition of aromatasee**
B001	MFLUCC15-1139	I (17%)	I (43%)	I (4%)	I (10%)	2.2	I (14%)
B002	MFLUCC15-1140	-	-	-	-	-	-
B003	MFLUCC15-1141	I (23%)	I (50%)	I (23%)	I (23%)	2.4	I (12%)
B004	MFLUCC15-1142	I (21%)	I (50%)	I (27%)	I (21%)	1.2	I (25%)
L001	MFLUCC15-1143	I (32%)	A (68%)	I (32%)	I (32%)	2.0	I (24%)
L002	MFLUCC15-1144	I (19%)	I (42%)	I (38%)	I (19%)	1.9	I (22%)
L003	MFLUCC15-1145	I (29%)	A (65%)	I (31%)	I (29%)	1.9	I (16%)
L004	MFLUCC15-1146	A (62%)	A (64%)	I (34%)	A (62%)	2.0	I (50%)
L005	MFLUCC15-1147	I (11%)	I (32%)	I (8%)	I (11%)	0.6	I (20%)
T001	MFLUCC15-1148	I (17%)	I (48%)	I (24%)	I (17%)	2.1	I (24%)
T002	MFLUCC15-1149	I (11%)	I (35%)	I (25%)	I (11%)	1.0	I (17%)
T003	MFLUCC15-1150	I (17%)	I (41%)	I (18%)	I (17%)	2.0	I (21%)
T004	MFLUCC15-1151	-	-	-	-	-	-
T005	MFLUCC15-1264	-	-	-	-	-	-
F001	MFLUCC15-1152	I (29%)	I (39%)	I (32%)	I (29%)	1.0	I (10%)
F002	MFLUCC15-1153	-	-	-	-	-	-
F003	MFLUCC15-1154	-	-	-	-	-	-

Note: I means inactive and A is active.

[a]DPPH is scavenging 2,2-diphenyl-1-picrylhydrazyl free radicals. Ascorbic acid was used as the reference compound with IC_{50} value of 21.2 μM.

[b]XXO is inhibition of superoxide anion radical formation by xanthine/xanthine oxidase and HL-60 is the inhibition of 12-*O*-tetra-decanoylphorbol-13-acetate-induced superoxide anion radical generation in differentiated HL-60 cell lines.

[c]IXO is inhibition of xanthine oxidase. The reference inhibitor is allopurinol with IC_{50} value of 3.0 μM.

[d]ORAC is oxygen radical absorbance capacity against $ROO^{\bullet}$ which represent in the ORAC unit. 1 ORAC unit equals the net protection of β-phycoerythrin produced by 1 μM Trolox

[e]Positive control in aromatase inhibition is ketoconazole with IC_{50} value of 2.4 μM.

Discussion

Isolation of endophytic fungi

The seventeen isolated endophytic fungi from *C. oleifera* were determined the genus based on their morphology. However, only six endophytic fungi can be identified at the genus level based on the fungal characteristic which were *Pestalotiopsis* sp. (MFLUCC15-1140, MFLUCC15-1141, MFLUCC15-1142, and MFLUCC15-1145), *Xylaria* sp. (MFLUCC15-1149 and MFLUCC15-1154), and *Collectotigum* sp. (MFLUCC15-1264) (Table 1).

Cytotoxic assay

The concentrations of the broth extracts from the endophytes were used at 30 μg/mL in the cytotoxicity studies. Extract of the unidentified endophytic fungus no. F001 (MFLUCC15-1152), was the only extract that exhibits cytotoxicity in HepG2 cell line, inhibiting at 55.73% by using MTT assay (IC_{50} of etoposide is 25.05 ± 3.06 μg/mL and IC_{50} of doxorubicin hydrochloride is 0.30 ± 0.02 μg/mL) [19]. Five broth extracts from the endophytic fungi, which are No. L004, L005, T001, T002, and F001 (MFLUCC15-1146, MFLUCC15-1147, MFLUCC15-1148, MFLUCC15-1149, and MFLUCC15-1152), showed cytotoxic activity more than 57% inhibition in Molt-3 cell lines (IC_{50} of etoposide is 0.041 ± 0.004 μg/mL) [19]. However, none of the broth extracts from the endophytic fungi showed inhibitory effects in HuCCA-1 and A549 cell lines which human lung cholangiocarcinoma and human lung carcinoma, respectively (Table 2).

Antioxidant assay

In the antioxidant assay, only the unidentified endophytic fungus No. L004 (MFLUCC15-1146) exhibits good activities in the radical scavenging, antioxidant, and aromatase inhibitory assay. It showed the activity to scavenge 2,2-dephenyl-1-picrylhydrazyl (DPPH) free radical (62% inhibition), inhibit superoxide anion radical formation in the xanthine/xanthine oxidase (XXO) assay (64% inhibition), inhibit the formation of TPA-induced superoxide anion radical in differentiated HL-60 human promyelocytic leukemia cell lines (62% inhibition), and show high ORAC antioxidant activity with 2.0 ORAC unit (Table 3).

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