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A new 5,6-dihydro-2-pyrone derivative from *Phomopsis amygdali*, an endophytic fungus isolated from hazelnut (*Corylus avellana*)

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ABSTRACT

Aims of this study were to isolate endophytes from different parts of hazelnut – *Corylus avellana* L. to obtain bioactive secondary metabolites and search for the presence of gene region of taxadiene synthase (Ts), a key enzyme in taxol biosynthesis, on selected fungi. Fourteen fungal species were isolated and cultured for the screening studies. The cell-free fermentation broths were extracted with chloroform. The chloroform extracts were tested for cytotoxic activity by MTT method. Based on the activity results and chemical profiles, the isolate identified as *Phomopsis amygdali* by internal transcribed spaces (ITS) sequence analysis using ITS1 primer was selected for further studies. After large-scale fermentation and purification studies, two major compounds, one of which turned out to be a new secondary metabolite, were isolated and characterized. Structure of the new metabolite was elucidated as (*S*)-4-butoxy-6-((*S*)-1-hydroxypentyl)-5,6-dihydro-2H-pyran-2-one by the extensive use of 1D and 2D NMR, and HR-MS, whereas the known compound was identified as (−)pestalotin. Additionally, to evaluate taxol-producing potential of the selected isolate, a PCR amplification study followed by gel electrophoresis analysis was carried out revealing no Ts gene region.

1. Introduction

The presence of as many as 1 million different fungal species has been estimated on our planet. In the past century, many have been described were those associated with various higher organisms as either parasites or saprophytes on biological materials. In the past few decades, plant scientists have begun to realize that plants may serve as a reservoir of untold numbers of organisms known as endophytes. These microorganisms mostly fungi and bacteria live in the intercellular spaces of plant tissues. Some of these endophytes produce bioactive substances that may be involved in a host–endophyte relationship (Hawksworth and Rossman, 1987; Strobel, 2003).

Since 1993, discovery of endophyte fungi *Taxomyces andreanae* and *Pestalotiopsis microspora* from *Taxus* sp. which produce the anticancer drug taxol, the endophytic fungi has been more attractive for many scientist. In subsequent years, it has been reported that all *Taxus* sp. harbor one or more endophytic fungi that produce taxol and taxol derivatives. After that, many studies focused on taxol-producing fungi, and most of the isolated fungi were also found capable to produce important bioactive secondary metabolites (Stierle et al., 1993; Zhao et al., 2010; Zhou et al., 2010). Moreover, it is expected that manipulation of endophytic fungi in laboratory to produce important secondary metabolites may lead to more reliable supplies of rare anticancer agent lead compounds of plant origin in the future (Pan et al., 2010).

Hazelnut fruit of *Corylus avellana* (Betulaceae) is known for its nutritional properties because of the special composition of fats, proteins, carbohydrates, vitamins (vitamin E), minerals and antioxidant phenolics (Alasalvar et al., 2003). Recent reports confirmed the availability of taxol and taxane derivatives such as 10-deacetylbaccatin III, baccatin III, paclitaxel C, and 7-epipaclitaxel in the shells and leaves of hazel plants (Bestoso et al., 2006; Hoffman and Shahidi, 2009; Ottaggio et al., 2008).

Thus, as a part of our ongoing studies to obtain cytotoxic agents from natural sources, we made an attempt to isolate endophytic fungi of hazelnut to purify novel bioactive secondary metabolites and show the presence of *Taxadiene synthase* (*Ts*) gene region that is the key enzyme in the biosynthesis of taxol. Different plant materials including the roots, branches and leaves were collected from Black Sea region of Turkey. After surface sterilization, inner

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tissues of the plant parts were placed on the surface of potato/dextrose/agar (PDA) medium and incubated at 28 °C, which resulted in isolation of 14 fungal species. The isolates were cultured in Malt Extract Broth medium at 150 rpm and 28 °C for 21 days in a rotary shaker. Cell-free fermentation broths were extracted with chloroform. The chloroform extracts were screened for cytotoxic activity by MTT method. Based on the cytotoxicity data, two fungal species [**R4** ($IC_{50} \leq 20$ μg/mL versus HT-29), and **L1** ($IC_{50} \leq 20$ μg/mL versus HT-29 and PC3)] were further characterized, and the isolate **L1** was found to be a more promising candidate.

2. Results and discussion

Although there are numerous reports on the endophytic fungi of different plants, to the best of our knowledge, there has been no study for *C. avellana*. This pioneering study resulted in the isolation of 14 morphologically different fungal species. While the leaves were found to be richest source (9 isolates), it was shown that the endophytes could be isolated from all parts of hazelnut. Two of the isolates, which were selected for further studies based on their chemical profiles and bioactivities, were identified as *Phomopsis amygdali* (**L1**) and *Fusarium oxysporium* (**R4**) by internal transcribed spaces (ITS) sequence analysis using ITS1 primer.

Although fungi belonging to the genus *Fusarium* are known as plant pathogens and soil contaminants, some of them have been demonstrated to produce important compounds, which were active against plant pathogens. Also some taxol producing *Fusarium* species from *Taxus* have been reported (Zhou et al., 2010).

The studies of fungi belonging to the genus *Phomopsis* has shown that these fungi could be rich source for various bioactive secondary metabolites such as antimicrotubule phomopsidin, antimalarial and antitubercular phomoxanthones, antifungal phomoxanthone A and phomodiol, herbicidal biraryl ethers, algicidal phomosines, cytokine production inhibitor phomalactone derivatives, antimicrobial phomopsichalasin and the plant growth regulator cytochalasin H (Horn et al., 1995; Strobel and Daisy, 2003; Rukachaisirikul et al., 2008; Nithya and Muthumary, 2011; Klaiklay et al., 2012). Also there are some *Phomopsis* species isolated from non-yew plants, reported to be able to produce anticancer drug taxol (Kumaran and Hur, 2009).

Chloroform extracts of the isolates **L1** and **R4** were tested against three cancer cell lines, namely PC-3 (human prostate adenocarcinoma-bone/CRL-1435TM), HT29 (human colon adenocarcinoma) and MDA-MB-231 (human breast adenocarcinoma) by MTT method. Both crude extracts demonstrated a moderate antitumor activity, whereas **L1** was slightly more active. Therefore the isolate **L1** was selected for further studies, which resulted in isolation of two compounds.

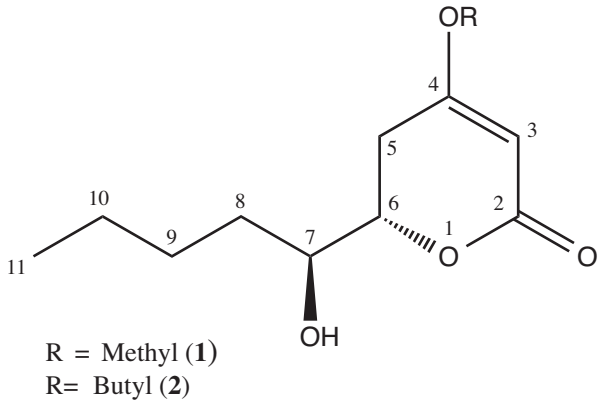

Fig. 1. Structures of **1** and **2**.

The NMR and MS spectral data of **1** were consistent with a 5,6-dihydro-2H-pyran-2-one. A detailed inspection of the 1D and 2D NMR spectra led to the identification of **1** as (*S*)-6-((*S*)-1-hydroxypentyl)-4-methoxy-5,6-dihydro-2H-pyran-2-one, a known compound named (−)pestalotin (Fig. 1) (Kimura et al., 1980; Kumar et al., 2004).

The molecular formula of **2** was determined as $C_{14}H_{24}O_4$ from HR-ESI-MS (*m/z* 257.2096 $[M+H]^+$) and the ^{1}H- and ^{13}C NMR data. Inspection of the ^{1}H NMR of **2** displayed two primary methyl groups (δ 0.91 and 0.95, each t and J = 7.2 Hz, H_3-11 and H_3-4′, respectively) in the upfield region, an olefinic (δ 5.11, d, J = 1.6 Hz, H-3), an oxymethylene (δ 3.89, m, H-1′), and two oxymethine protons (δ 3.61, ddd, J = 4.4, 4.4, 8.0 Hz; δ 4.28, dt, J = 4.0, 13.2 Hz, H-7 and H-6, respectively) in the downfield region. In the ^{13}C NMR spectrum of **2**, the oxymethylene and oxymethyn carbons were readily deduced at δ 69.2 (C-1′), 72.7 (C-7) and 78.5 (C-6), confirmed by the cross peaks in the HMQC spectrum. In the downfield region, the remaining three additional carbons (δ 172.6, 167.1 and 90.3) were significant for one carbonyl group and an olefinic system, demonstrating two of three unsaturation inferred from the molecular formula (Table 1). Thus compound **2** was deduced to be a monocyclic compound.

The combined use of the COSY and HMQC spectra allowed the assignment of two spin systems (Fig. 2): [H-3 (allylic coupling) → H-5 → H-11] and [H-1′ → H-4′]. The first spin system was completely identical with pestalotin's 4-hydroxy-6-(1-hydroxypentyl)-5,6-dihydro-2H-pyran-2-one framework. The second

Table 1
^{13}C and ^{1}H data of compound **2** (400 MHz, $CDCl_3$)[a], (−)pestalotin (400 MHz, $CDCl_3$) and (+)pestalotin (360 MHz, $CDCl_3$).

C/H	**2**		(−)Pestalotin	(+)Pestalotin
	δ_C	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
2	167.1			
3	90.3	5.11 (d, 1.6)	5.14 (s)	5.35 (d, 1.1)
4	172.6			
5	29.8	2.76 (ddd, 1.6, 12.8, 16.8), 2.26 (dd, 3.7, 17.2)	2.82 (dd, 12, 16.6), 2.24 (dd, 4, 16.6)	3.04 (ddd, 2.5, 5.4, 18.6), 3.52 (d, 18.6)
6	78.6	4.28 (dt, 4.0, 13.2)	4.28 (m)	4.37 (m)
7	72.7	3.61 (ddd, 4.4, 4.4, 8.0)	3.60 (m)	4.16 (m)
8	32.6	1.59	2.1	2.07
9	27.8	1.43	1.68	1.80
10	22.8	1.33	1.28	1.39
11	14.2	0.91 (t, 7.2)	0.96 (t, 7.4)	0.94 (t, 6.7)
1′	69.2	3.89 (m)	3.79 (s)	3.66 (s)
2′	30.6	1.73 (m)	–	–
3′	19.3	1.45 (m)	–	–
4′	13.6	0.95 (t, 7.2)	–	–

[a] Assignments confirmed by COSY, HMQC and HMBC experiments.

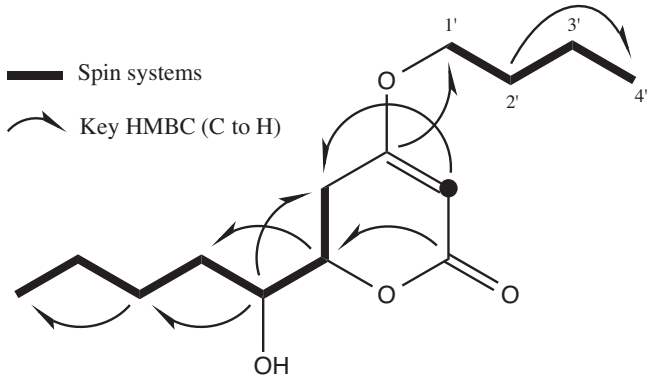

Fig. 2. Spin systems deduced from COSY and key long correlations from HMBC.

spin system implied that compound **2** had an *O*-butyl moiety. To associate these two fragments, a long-range $^{1}H–^{13}C$ NMR measurement was performed. Based on the correlation (Fig. 2) between H-1′ (δ 3.89) and C-4 (δ 172.6), the planar structure of **2** was elucidated as 4-butoxy-6-(1-hydroxypentyl)-5,6-dihydro-2H-pyran-2-one, a new structure.

The stereochemistry of **2** was resolved by comparing optical rotation data [**2**: $[\alpha]_D^{25} = -42.1$, c 0.0019 $CHCl_3$; (−)pestalotin: $[\alpha]_D^{25} = -86.2$, c 0.14, MeOH; (+)pestalotin: $[\alpha]_D^{25} = +91.1$, c 1.34, MeOH] and NMR chemical shifts/coupling constant values (Table 1) of **2** with those of known compounds, which clearly supported absolute configuration at the stereocenters as C-6(*S*) and C-7(*S*) (Wang and Shen, 1997; Mayer et al., 2002; Kumar et al., 2004).

As far as it is ascertained, 5,6-dihydro-2H-pyran-2-one derivatives were encountered for the first time in *Phomopsis* genus.

Compounds **1** and **2** were also tested against three cancer cell lines including breast, prostate and colon (MDA-MB-231, PC-3 and HT-29) and a normal cell line (HEK293). The results are presented in Table 2. Both compounds showed moderate cytotoxicity toward cell lines compared to positive control doxorubicin. In particular, compound **1** had superior activity than **2** against cancer cell lines PC-3 (ca. 6-fold) and HT-29 (ca. 5-fold) (IC_{50}: 20.54, 16.69 μg/mL, respectively), whereas **2** was more active versus MDA-MB-231 cell line with IC_{50} value of 24.26 μg/mL.

Biosynthesis of taxol and related taxoids is initiated from cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene by catalysis of *Taxadiene synthase* (*Ts*) enzyme. Thus this is the first step in constructing the unique taxane skeleton (Jennewein and Croteau, 2001). In this study, we also searched for any gene matching with *Ts* region by PCR amplification to evaluate taxol-producing potential of the isolates **L1** and **R4**. Following, approximately 400 bp products were obtained, whereas it was expected to be 632 bp for *Ts* gene. The PCR results revealed no *Ts* gene region for both isolates. Although *Ts* is the rate-limiting enzyme, it is not the only enzyme in the taxol biosynthesis. Subsequently more than twenty steps take place in taxol biosynthesis.

Table 2
Cytotoxic activities of **1** and **2**.[a]

	IC_{50} (μg/mL)			
Cell lines	PC-3	HT-29	MDA-MB-231	HEK 293
Compounds				
1	20.54	16.69	41.70	11.83
2	132.23	82.18	24.26	13.84
Doxorubicin	2.67	1.99	3.48	1.01

[a] These values are means of three independent experiments. Doxorubicin was used as positive control, and 1% DMSO was negative control for the assays.

In conclusion, based on previous reports indicating that some taxol producing fungi might have different biosynthetic pathway for taxol production (Zhang et al., 2008), it is not rational to conclude that tested fungal strains are not taxol producers. Further studies coupled with LC–MS and NMR techniques are warranted to clarify this matter.

3. Experimental

3.1. General experimental procedures

Potato Dextrose Agar (PDA, Sigma), Malt Extract Agar (MEA, Sigma), Rose Bengal Agar (RB, Sigma) and Actinomycete Isolation Agar (AIO, Sigma) were used for the isolation of fungal endophytes; Malt Extract Broth (MEB; Merck) was used as fermentation broth; Incubated Shaker (Gerhardt VTO500) was used for fermentation; Silica gel 230–400 mesh (Merck); reverse-phase material (C-18, Sepralyte 40 μm) were used for vacuum liquid chromatography; pre-coated silica gel GF254 aluminum sheets (Merck) were used for thin layer chromatography (TLC) with *n*-hexane:ethyl acetate:methanol (10:10:3); visualization was performed by spraying 30% sulfuric acid (H_2SO_4); the following equipment and conditions were also used: Heidolph Laborota 4001 rotavapor; Christ-ALPHA 1-4 LD freeze dryer; CAMAG UV Lamp, Welch 2511 vacuum pump. Optical rotation (at λ = 598 nm) was measured on a Rudolph Research Autopol I polarimeter with a 1-mL capacity quartz cell (0.25 dm path length). NMR spectra were obtained on a Varian Mercury Plus 400 spectrometer in *d*-chloroform ($CDCl_3$) at 30 °C. The PCR amplification was performed in a Techne TC-3000X thermal cycler. The cell lines were maintained in RPMI 1640 (Biochrom, Germany).

3.2. Isolation and identification of fungal strains

The endophytic fungus was isolated from the parts of *C. avellana*, collected in November 2010 from Black Sea Region (Ordu-Akçatepe Village; Deposition number: IZEF-5965), Turkey. Different plant parts including the roots, branches and healthy leaves were obtained and surface sterilized with 3% NaOCl. First, plant materials rinsed with distilled water and thoroughly surface treated with 70% ethanol. Subsequently, samples dipped into 3% NaOCl for 5 min, and rinsed with sterile water, then allowed to surface-dry under a laminar-flow hood. The last washing water was incubated to test the surface sterilization. The bark layers were removed with a sharp, sterilized blade, and small inner tissue pieces were placed on the surface of PDA plates in laminar flow cabinet and then incubated at 24–28 °C for 5 days. Subsequently 14 representative fungal species were subcultured in new PDA plates to obtain pure isolates. Fermentation for all fungal isolates was performed in 1000 mL flask containing 250 mL of MEB medium, and incubated at 150 rpm and 28 °C for 21 days in a rotary shaker. Then the fermentation broths were extracted with chloroform, and chloroform extracts were screened for their chemical profiles by TLC. Leaves were the richest part of the plant in terms of number of isolates (9 isolates). Among 14 representative isolates, **L1** and **R4** were selected for further studies and identified. **L1** colony, obtained from hazelnut leaves, grew slowly on PDA, and exhibited white color, while demonstrating a very viscous growth in liquid medium (see Supporting information). The other endophyte fungus **R4** was isolated from inner tissue of the hazelnut roots. The colony of the isolate **R4** was initially white and slowly turned to pale pink on PDA medium after several days of fermentation.

Both traditional morphological assessment and internal transcribed spaces (ITS) sequence analysis were performed to characterize the fungal strains. First high molecular weight genomic DNA was isolated from the strains (Liu et al., 2000).

ITS1 primer (5′-TCCGTAGGTGAACCTGCGG-3′ was used to identify of **R4** and **L1** isolates/strains. Based on the ITS sequences of the isolates, the endophyte fungi **L1** and **R4** were identified as *P. amygdali* and *F. oxysporium*, respectively (see Supporting information).

3.3. TLC analysis and separation

All crude extracts were applied to TLC plate to view their chemical contents. On the basis of TLC profiles and cytotoxic activity results, the isolate **L1** was selected for further studies. Strain **L1** was cultured with MEB medium in 1000 mL Erlenmeyer flasks containing 300 mL medium and incubated for three weeks. The fermentation broth (5000 mL) was filtered through cheesecloth, and the filtrate was extracted with chloroform (4× 500 mL) to afford a brown gummy extract (58.3 mg). The extract was applied to vacuum liquid chromatography (VLC) on reversed-phase material (RP-18, 20 g) employing H_2O (150 mL), H_2O–MeOH (80:20, 50 mL; 60:40, 300 mL; 40:60, 250 mL; 20:80, 150 mL), and MeOH (200 mL) to yield four fractions; L1F0 (6.7 mg), L1F1 (7 mg), L1F2 (16.3 mg), L1F3 (21.5 mg). After TLC controls, fractions L1F2 (compound **1**) and L1F3 (compound **2**) were obtained as pure substances

3.4. Cytotoxic activity

Screening of the crude extracts and pure substances (**1** and **2**) for cytotoxicity, based on metabolic cell viability, was carried out by using a modified MTT 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Urgen et al., 2010). Cytotoxicity assay was performed against three cancer cell lines; PC-3 (human prostate adenocarcinoma-bone/CRL-1435TM), HT-29 (human colon adenocarcinoma) and MDA-MB-231 (human breast adenocarcinoma), and a normal cell line HEK293 (human embryonic kidney 293).

3.5. Screening of Ts gene site by PCR amplification

The DNA extraction from endophytic fungi was performed with method described by Liu et al. (2000). Subsequently isolates were screened based on conserved sequence of Ts gene (Gene Bank: AY364469) by PCR amplification. The specific primers TsF (5′-CAAACCCATGTCGAATTGAG-AAG-3′) and TsR (5′-CAAGTTTGCATACACTC-TGGAATCT-3′) were used. The PCR amplification was performed for 32 cycles (94 °C for 50 s, 55 °C for 1 min, and 72 °C for 80 s) followed by extension for 7 min at 72 °C (Zhou et al., 2007). The PCR products were analyzed by gel electrophoresis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2013.09.012.

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