Regular article

Production of small molecules by an endophytic fungus, *Neofusicoccum parvum* from the fruits of *Elaeocarpus serratus*

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Abstract

***Neofusicoccum parvum*, an endophytic fungus isolated from *Elaeocarpus serratus* (Ceylon Olive; family Elaeocarpaceae), was grown for 3 weeks in potato dextrose broth. Chromatographic separation of the ethyl acetate extracts from the culture filtrate and mycelium over silica gel, Sephadex LH-20 and preparative thin layer chromatography furnished (*R*)-7-hydroxymellein (1), (3*R*,4*R*)-4-hydroxymellein (2), (3*R*,4*S*)-4-hydroxymellein (3), (*R*)-5-hydroxymellein (4), (*R*)-mellein (5), (3*R*,4*R*)-4,7-dihydroxymellein (6), (6*R*,7*S*)-dia-asperlin (7), CJ-14445 (8) and 13,14,15,16-tetranorlabd-7-ene-19,6*β*:12,17-diolide (9). The structures of known compounds 1–9 were determined by spectroscopic methods and comparison with reported data. This is the first report of the isolation of an endophytic fungus from *E. serratus*, and the isolation of compounds 1, 4, 6, 8 and 9 from *N. parvum*. It is important to note that compounds 1–7 are small molecules with an oxygen heterocyclic ring system. These compounds can be used as starting materials in the synthesis of pharmaceutically important large molecules with oxygen heterocyles.**

***Key words*: *Neofusicoccum parvum*; *Elaeocarpus serratus*; endophytic fungi; secondary metabolites; dihydroisocoumarines**

1 Introduction

Natural products from microorganisms play a major role in agriculture, medicine, pharmaceutical, and food industry due to their bioactivity. Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically remained unnoticed [1]. These years fungal endophytes have been attracting the interest as sources of novel bioactive metabolites. We have previously reported several bioactive compounds produced by fungi isolated from Sri Lankan plants [2-13]. In a continuation of our studies, chemical investigation of secondary metabolites produced by an endophytic fungus *Neofusicoccum parvum* isolated from *Elaeocarpus serratus* (Ceylon Olive; family Elaeocarpaceae) has been carried out.

Fruits of *E. serratus* are edible and popular

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in Sri Lanka. Water extracts of the leaves of *E. serratus* are used in anti-head lice and anti-dandruff treatments in rural areas of Sri Lanka. We reported previously the isolation of flavonoids, myricitrin, mearnsitrin, mearnsetin 3-*O*-*β*-D-glucopyranoside and tamarixetin 3-*O*-*α*-L-rhamnopyranoside from *E. serratus*, and their antioxidant activity [14]. Only a few papers have been published on the secondary metabolites produced by *N. parvum* [15,16].

2 Experimental

2.1 General

Extractions were performed using a sonicator (VWR Ultrasound cleaner, model-USC 1700 D). TLC analysis was conducted on silica gel plates (Merck 1.05554, $60F_{254}$, 0.20 mm thickness). TLC spots were located using a UV lamp and by heating after spraying with acidic anisaldehyde. Silica gel (Merck Art. 7734 & 9385) and Sephadex LH-20 were used for column chromatography. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-AL300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) spectrometer in $CDCl_3$ or CD_3OD solution with Me_4Si as the internal standard. ESIMS were obtained on a Thermo Scientific LCQ Fleet MS Instrument.

2.2 Isolation and identification of the endophytic fungus

Fresh, healthy and matured fruits of *Elaeocarpus serratus* were collected from Central Province of Sri Lanka. Fruits were washed with running tap water and triple sterilized with 2% sodium hypochlorite (NaOCl), 75% ethanol and finally washed with sterile distilled water. A few segments of the inner fleshy part of the fruits were placed on potato dextrose agar (PDA) medium and allowed to stand at room temperature for 4–7 d in the dark. The emerging fungus was subcultured serially to obtain a pure culture of the fungus, which was identified as *Neofusicoccum parvum* by sequence analysis of ITS1 and ITS4 regions of the rDNA gene. BLAST search indicated that the sequence of the ITS region showed 100% similarity to that of *N. parvum* (GenBank Accession No. KR260805.1). Fungal identification was carried out by GeneTech, Colombo, Sri Lanka. Photographic evidences of the fruits of *E. serratus* and *N. parvum* strain (IFS/DMESA/2016) are deposited at the National Institute of Fundamental Studies.

2.3 Fermentation, extraction and isolation

1 L Erlenmeyer flasks (x 60) each containing PDB medium (400 mL) were inoculated with *N. parvum*. The flasks were incubated at room temperature for 21 d. After 10 d of undisturbed incubation, the flasks were shaken at 100 rpm on a laboratory shaker for 2 h per d until extraction. The medium was filtered and the filtrate was extracted with ethyl acetate (EtOAc). The mycelium was crushed into small pieces and extracted into EtOAc followed by MeOH. Removal of the solvents on a rotary evaporator afforded the respective extracts. The TLC patterns of EtOAc extracts of the culture broth and mycelium were found to be almost similar. Hence the extracts were combined together. Both EtOAc and MeOH extracts were subjected to bioassays for antifungal activity against *Cladosporium cladosporioides* [17], antioxidant activity against DPPH free radicals [18], phytotoxic activity against lettuce seed (*Lactuca sativa*) germination [19]. The combined EtOAc extract (8.4 g) was chromatographed over silica gel Sephadex LH-20 and PTLC to furnish compounds **1** (10 mg), **2** (4 mg), **3** (4 mg), **4** (5 mg), **5** (9 mg), **6** (15 mg), **7** (15 mg), **8** (15 mg) and **9** (15 mg).

2.3.1 (R)-7-Hydroxymellein (***1***)

White solid; $[\alpha]_D^{25}$ -86.4 (*c*, 0.11, $CHCl_3$) (lit. $[\alpha]_D^{20}$ -97 ($CHCl_3$) [20]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 11.06 (1H, *s*, 8-OH), 7.08 (1H, d, *J* = 7.8 Hz, H-5), 6.62 (1H, d, *J* = 7.8 Hz, H-6), 5.58 (1H, s, 7-OH), 4.74 (1H, m, H-3), 2.88 (2H, d, *J* = 7.5 Hz, H_2-4), 1.53 (3H, d, *J* = 6.3 Hz, H_3-9); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 170.2 (C-1), 149.0 (C-8), 143.7 (C-7), 129.7 (C-4a), 120.5 (C-6), 117.6 (C-5), 108.1 (C-8a), 77.1 (C-3), 33.9 (C-4), 20.7 (C-9).

2.3.2 (3R,4R)-4-Hydroxymellein (***2***)

Colorless needles; $[\alpha]_D^{25}$ -40.5 (*c*, 0.70, MeOH) (lit. $[\alpha]_D$ + 37.4 (*c*, 0.33, MeOH, for (+)-(*S*)-**2**) [21]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 10.99 (1H, s, 8-OH), 7.53 (1H, dd, *J* = 8.4, 7.3 Hz, H-6), 7.04 (1H, dd, *J* = 8.4, 1.2 Hz, H-7), 6.93 (1H, dd, *J* = 7.3, 1.2 Hz, H-5), 4.70 (1H, qd, *J* = 6.6, 1.6 Hz, H-3), 4.57 (1H, d, *J* = 1.6 Hz, H_2-4), 2.08 (1H, br, 4-OH), 1.59 (3H, d, *J* = 6.6 Hz, H_3-9); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 170.2 (C-1), 149.0 (C-8), 143.7 (C-7), 129.7 (C-4a), 120.5 (C-6), 117.6 (C-5), 108.1 (C-8a), 77.1 (C-3), 33.9 (C-4), 20.7 (C-9). ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 169.2 (C-1), 162.0 (C-8), 140.4 (C-4a), 136.8 (C-6), 118.5 (C-7), 118.3 (C-5), 106.8 (C-8a), 78.2 (C-3), 67.2 (C-4), 16.0 (C-9).

2.3.3 (3R,4S)-4-Hydroxymellein (***3***)

Colorless needles; $[\alpha]_D^{25}$ -5.9 (*c*, 0.5, MeOH) (lit. $[\alpha]_D$ -29 (MeOH) [22]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 10.99 (1H, s, 8-OH), 7.55 (1H, dd, *J* = 8.4, 7.7 Hz, H-6), 7.02 (1H, brd, *J* = 7.7 Hz, H-5), 6.99 (1H, brd, *J* = 8.4 Hz, H-7), 4.67-4.56 (2H, m, H-3, H_2-4), 2.33 (1H, br, 4-OH), 1.53 (3H, d, *J* = 6.3 Hz, H_3-9); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: 170.2 (C-1), 149.0 (C-8), 143.7 (C-7), 129.7 (C-4a), 120.5 (C-6), 117.6 (C-5), 108.1 (C-8a), 77.1 (C-3), 33.9 (C-4), 20.7 (C-9). ^{13}C-NMR ($CDCl_3$): δ 168.5 (C-1), 162.0 (C-8), 141.1 (C-4a), 136.9 (C-6), 117.8 (C-7), 116.2 (C-5), 106.6 (C-8a), 79.9 (C-3), 69.1 (C-4), 17.9 (C-9).

2.3.3.1 Absolute stereochemistry of compound (***3***)

Compound **3** was converted to 4-*O*-(*S*)- and 4-*O*-(*R*)-MTPA esters by reaction with (*R*)- and (*S*)-MTPA chlorides, respectively, along with the respective di-MTPA esters. These MTPA esters showed a single set of proton signals in the ^{1}H NMR, thus compound **3** was established to be optically active even though the $[\alpha]_D$ value of **3** was considerably smaller than the reported one. ^{1}H NMR (300 MHz, $CDCl_3$) of the 3-*O*-(*S*)-MTPA ester: δ 10.93 (1H, *s*, 8-OH), 7.51 (1H, dd, *J* = 8.4, 7.7 Hz, H-6), 7.07 (1H, brd, *J* = 8.4 Hz, H-5), 6.90 (1H, brd, *J* = 7.7 Hz, H-7), 5.97 (1H, d, *J* = 3.2 Hz, H-4), 4.96 (1H, qd, *J* = 6.6, 3.2 Hz, H-3), 3.51 (3H, brd, *J* = 1.5 Hz, CH_3O), 1.43 (3H, d, *J* = 6.6 Hz, H_3-9). ^{1}H NMR (300 MHz, $CDCl_3$) of the 3-*O*-(*R*)-MTPA ester: δ 11.00 (1H, s, 8-OH), 7.54 (1H, dd, *J* = 8.4, 7.7 Hz, H-6), 7.10 (1H, brd, *J* = 8.4 Hz, H-5), 6.97 (1H, brd, *J* = 7.7 Hz, H-7), 5.99 (1H, d, *J* = 3.2 Hz, H-4), 4.56 (1H, qd, *J* = 6.6, 3.2 Hz, H-3), 3.40 (3H, brd, *J* = 1.5 Hz, CH_3O), 1.40 (3H, d, *J* = 6.6 Hz, H_3-9). The absolute configuration at C-3 of compound **3** was determined to be *S* based on $\Delta(\delta(S)\text{-}\delta(R))$ values by application of Mosher's ester method [23]. The result confirmed the 3*R*,4*S*-configuration of **3**, which was originally proposed by assuming compound **3** and **4** could be biosynthetically correlated to (-)-(*R*)-mellein [22]. Absolute stereochemistry of (+)-(3*S*,4*R*)-4-hydroxymellein, which was deduced from observed and calculated ECD spectra has been reported [24].

2.3.4 (R)-5-Hydroxymellein (***4***)

White solid; $[\alpha]_D^{25}$ -77.2 (*c*, 0.42, MeOH) (lit. $[\alpha]_D$ -72 (*c*, 1.65) [25]; ^{1}H-NMR (300 MHz,

CD_3OD): δ 7.01 (1H, d, J = 8.7 Hz, H-6), 6.69 (1H, d, J = 8.7 Hz, H-7), 4.69 (1H, m, H-3), 3.16 (1H, dd, J = 16.8 Hz, 3.3, Ha-4), 2.62 (1H, dd, J =2 16.8, 11.4 Hz, Hb-4), 1.50 (3H, d, J = 6.3 Hz, H_3-9); ^{13}C-NMR (75 MHz, CD_3OD): δ 171.7 (C-1), 156.3 (C-8), 146.8 (C-5), 125.7 (C-4a), 125.1 (C-6), 116.5 (C-7), 109.1 (C-8a), 77.6 (C-3), 29.4 (C-4), 21.1 (C-9).

2.3.5 (R)-Mellein (***5***)

White amorphous solid; $[\alpha]_D^{25}$ -93.3 (*c*, 1.15, $CHCl_3$) (lit. $[\alpha]_D^{21}$ -94.4 (*c*, 1.05, $CHCl_3$) [26]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 11.03 (1H, s, 8-OH), 7.41 (1H, d, J = 8.0 Hz, H-6), 6.89 (1H, d, J = 8.0 Hz, H-7), 6.69 (1H, d, J = 8.0 Hz, H-5), 4.74 (1H, dq, J = 6.9, 6.6 Hz, H-3), 2.93 (2H, d, J = 6.9 Hz, H_2-4), 1.53 (3H, d, J = 6.6 Hz, H_3-9); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 169.9 (C-1), 162.1 (C-8), 139.4 (C-4a), 136.1 (C-6), 117.9 (C-5), 116.2 (C-7), 108.2 (C-8a), 76.1 (C-3), 34.6 (C-4), 20.7 (C-9).

2.3.6 (3R,4R)-4,7-Dihydroxymellein (***6***)

White amorphous solid; $[\alpha]_D^{25}$ -31.6 (*c*, 0.24, MeOH) (lit. $[\alpha]_D^{28}$ -70.5 (*c*, 0.13, MeOH) [27]; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 11.09 (1H, s, 8-OH), 7.17 (1H, d, J = 8.1 Hz, H-6), 6.87 (1H, d, J = 8.8 Hz, H-5), 5.73 (1H, br, 7-OH), 4.70 (1H, dq, J = 6.6, 1.8 Hz, H-3), 4.54 (1H, brs, H-4), 1.68 (1H, br, 4-OH), 1.59 (3H, d, J = 6.6 Hz, H_3-9); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 169.4 (C-1), 149.0 (C-8), 145.7 (C-7), 131.2 (C-4a), 120.8 (C-6), 118.6 (C-5), 106.8 (C-8a), 79.1 (C-3), 66.9 (C-4), 16.2 (C-9).

2.3.7 (6R,7S)-Dia-asperlin (***7***)

White crystalline solid; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.87 (dd, J = 9.6, 5.0 Hz, H-3), 6.23 (1H, d, J = 9.6 Hz, H-2), 5.51 (1H, dd, J = 5.0, 3.9 Hz, H-4), 4.36 (1H, dd, J = 4.8, 3.9 Hz, H-5), 3.11-2.99 (2H, m, H-6, H-7), 2.16 (3H, s, CH_3CO), 1.35 (3H, d, J = 5.1 Hz, H_3-8); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 170.0 (CH_3CO), 161.5 (C-1), 139.8 (C-3), 125.1 (C-2), 78.2 (C-5), 63.0 (C-4), 56.7 (C-6), 50.6 (C-7), 20.7 (CH_3CO), 17.1 (C-8).

2.3.8 CJ-14445 (***8***)

White crystalline solid; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.22 (1H, m, H-7), 5.78 (1H, d, J =1.5 Hz, H-11), 5.04 (1H, m, H-6), 5.00 (1H, brd, J =13.5 Hz, H-13a), 4.89 (1H, d, J = 13.5 Hz, H-13b), 2.27 (1H, m, H-3a), 1.95 (1H, d, J = 4.2 Hz, H-5), 1.87 (1H, m, H-2a), 1.35 (3H, s, H_3-16), 1.19 (3H, s, H_3-14), ^{13}C-NMR (75 MHz, $CDCl_3$): δ 180.9 (15-CO), 163.7 (12-CO), 158.7 (C-9), 132.2 (C-8), 121.8 (C-7), 111.8 (C-11), 71.3 (C-6), 69.6 (C-13), 47.8 (C-5), 42.8 (C-4), 35.0 (C-10), 29.6 (C-1), 27.7 (C-3), 24.7 (C-14), 24.1 (C-16), 17.3 (C-2).

2.3.9 13,14,15,16-Tetranorlabd-7-ene-19,6β:12,17-diolide (***9***)

White crystalline solid; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.07 (1H, m, H-7), 4.97 (1H, m, H-6), 4.84 (1H, d, J = 14.7 Hz, Ha-13), 4.76 (1H, d, J = 14.7 Hz, Hb-13), 2.64 (1H, m), 2.46-2.32 (2H, m), 2.22-2.11 (1H, m), 1.82 (1H, d, J = 5.1 Hz, H-5), 1.82-1.49 (4H, m), 1.35 (3H, s, H_3-14), 1.26 (1H, dd, J = 12.0, 6.0 Hz), 0.88 (3H, s, H_3-16), ^{13}C-NMR (75 MHz, $CDCl_3$): δ 181.3 (15-CO), 172.4 (12-CO), 138.4 (C-8), 119.1 (C-7), 72.2 (C-6), 69.8 (C-13), 51.1 (C-9), 44.8 (C-5), 42.4 (C-4), 33.5 (C-10), 32.2 (C-11), 29.1 (C-1), 27.7 (C-3), 24.1 (C-16), 17.5 (C-14), 17.4 (C-2).

3 Results and Discussion

An endophytic fungus was isolated from the fruits of *E. serratus* and identified as *Neofusicoccum parvum* based on the sequence

of the internal transcribed spacer (ITS) region of rDNA. Fermentation of *N. parvum* was carried in potato dextrose broth (PDB) for 3 weeks. The culture filtrate and the mycelium were individually extracted with EtOAc. The combined EtOAc extract showed antifungal activity against *Cladosporium cladosporioides*, DPPH radical scavenging activity (IC_{50} 56 μg/mL) and phytotoxic activity against lettuce seed germination by inhibiting root and shoot elongation (IC_{50} 219 and 242 μg/mL, respectively). Chromatographic separation of the EtOAc extract over silica gel, Sephadex LH-20 and preparative TLC furnished nine previously known metabolites **1**–**9** (Fig. 1). The structures of compounds **1**–**9** were determined by spectroscopic methods and comparison with reported data: (*R*)-7-hydroxymellein (**1**) [27], (3*R*,4*R*)-4-hydroxymellein (**2**) [28,29], (3*R*,4*S*)-4-hydroxymellein (**3**) [28], (*R*)-5-hydroxymellein (**4**) [30], (*R*)-mellein (**5**) [31], (3*R*,4*R*)-4,7-dihydroxymellein (**6**) [27], (6*R*,7*S*)-dia-asperlin (**7**) [15], CJ-14445 (**8**) [32,33] and 13,14,15,16-tetranorlabd-7-ene-19,6*β*:12,17-diolide (**9**) [34]. Compounds **5**, **6** and **7** showed good antifungal activity *against C. cladosporioides*.

It is noticeable that six compounds out of the nine metabolites belong to the family of mellein-type dihydroisocoumarins. Their configurations at C-3 were found to be *R* consistently as revealed by comparison of their specific rotations with literature values. (*R*)-7-hydroxymellein (**1**) and (3*R*,4*R*)-4,7-dihydroxymellein (**6**) were previously isolated from a few fungi such as an endophytic *Penicillium* sp. and their antifungal properties against *C. cladosporioides* and *C. spahaerospermum* and acetylcholinesterase inhibitory activity were reported [27]. (3*R*,4*R*)- and (3*R*,4*S*)-4-hydroxymelleins (**2** and **3**) have been isolated, occasionally as a diastereomeric pair, from several fungal sources including the phytopathogenic fungus *Septoria nodorum* [25] and also from an Annonaceous plant, *Uvaria hamiltonii* [28]. It was reported that compound **2** showed phytotoxicity causing wilting of leaves when assayed on tomato cuttings [16], whereas compounds **2** and **3** displayed cytotoxic activities against Hela, DU145, U937 and HL60 cell lines [35]. Antifungal, antibacterial and algicidal properties of (*R*)-mellein (**5**) were reported [36]. In addition, herbicidal activity of **5** has recently been reported [37]. Isolation of (6*R*,7*S*)-Dia-asperlin (**7**) was reported from limited fungal sources like *N. parvum* [14] and *Aspergillus* sp. [38]. CJ-14445 (**8**) was reported to show a variety of bioactivities including inhibition of IL-1β and

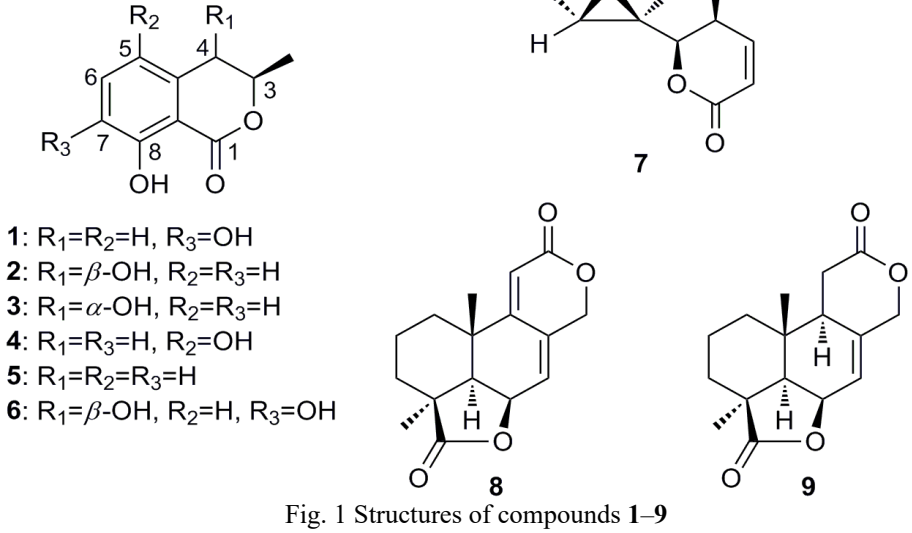

Fig. 1 Structures of compounds **1**–**9**

TNF-α production [32], herbicidal, antiplasmodial and cytotoxic activities [39], growth inhibition of several human cancer cell lines and antifungal activity against *Cryptococcus neoformans* and *Candida albicans* [33]. 13,14,15,16-Tetranorlabd-7-ene-19,6*β*:12,17-diolide (**9**) was found to have a potent cytotoxic activity against MCF-7 cell line [40]. *N. parvum* has been found associated with a wide range of tree species, including grapevine, fruit and forest trees, and shown to be responsible for diseases on various trees and shrubs [41].

4 Conclusion

Among the 9 compounds, five compounds **1**, **4**, **6**, **8** and **9** were isolated from *N. parvum* for the first time, whereas the rest of compounds were previously reported from *N. parvum* [15,16]. It is important to note that compounds **1**–**7** are small molecules with an oxygen heterocyclic ring system. These compounds can be used as starting materials in the synthesis of pharmaceutically important large molecules with oxygen heterocyles. Further this study proves that the endophytic fungus *N. parvum* isolated from the fruits of *E. serratus* is a good source for simple oxygen heterocyclic compounds.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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