Fitoterapia 167 (2023) 105496

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Antibacterial eremophilane sesquiterpenoids from *Xylaria feejeensis*, an endophytic fungi of the medicinal plant *Geophila repens*

Sanjeevan Rajendran [a,b], Luke P. Robertson [a], Lakmini Kosgahakumbura [a,b], Chathurika Fernando [b], Ulf Göransson [a], Helen Wang [c], Chamari Hettiarachchi [b], Sunithi Gunasekera [a,*]

[a] Pharmacognosy, Department of Pharmaceutical Biosciences, Biomedical Center, Uppsala University, Sweden
[b] Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka
[c] Department of Medical Biochemistry and Microbiology, Biomedical Centre, Uppsala University, Sweden

ARTICLE INFO

Keywords:
Endophytic fungi
Antibacterial
Geophila repens
Xylaria feejeensis
Eremophilane Sesquiterpenoids

ABSTRACT

Geophila repens (L.) I.M. Johnst (Rubiaceae) is a traditional medicinal plant used in Sri Lanka for the treatment of bacterial infections. Due to its rich endophytic fungi content, it was postulated that endophytically-produced specialized metabolites may be responsible for its purported antibacterial effects. To test this hypothesis, eight pure endophytic fungal cultures were isolated from *G. repens* then extracted and screened for antibacterial activity in a disc diffusion assay against *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa*. Large scale culturing, extraction, and purification of the most active fungal extract, obtained from *Xylaria feejeensis*, led to the isolation of 6′,7′-didehydrointegric acid (**1**), 13-carboxyintegric acid (**2**), and four known compounds including integric acid (**3**). Compound **3** was isolated as the key antibacterial component (MIC = 16 μg/mL against *Bacillus subtilis*, 64 μg/mL against Methicillin-Resistant *S. aureus*). Compound **3** and its analogues were devoid of hemolytic activity up to the highest tested concentration of 45 μg/mL. This study demonstrates that specialized metabolites produced by endophytic fungi may contribute to the biological activity of some medicinal plants. Endophytic fungi should be evaluated as a potential source of antibiotics, especially from unexplored medicinal plants traditionally used for the treatment of bacterial infections.

1. Introduction

The emergence of drug resistant microbes is one of the great challenges of modern medicine [1]. Sri Lanka, being a biodiversity hot spot, has a rich collection of plants that have been used in traditional medicine for over 3000 years. Sri Lanka contains c.a. 3154 indigenous flowering plant species, of which 894 are endemic [2]. Both host plants and microbes found in Sri Lanka show a high rate of endemic speciation [3]. Consequently, the presence of potentially unique biosynthetic pathways offers opportunities to discover structurally diverse specialized metabolites with new biological activities [4].

While many plants are used in Sri Lankan traditional medicine, anecdotal evidence of their usage is often not supported by strong scientific research, and the chemistry of the underlying bioactive molecules remains elusive. Emerging studies have indicated that the organisms that colonize the interior of the plants may play a vital role in plant defense and specialized metabolite production. In particular, plants that endure harsh biotic and abiotic stresses have been a promising source of endophytic fungi enriched with pharmacologically active metabolites [5–7]. Among the many antibacterial leads found in endophytic fungi, many structurally diverse chemical compounds (e.g., alkaloids, peptides, polyketides, terpenoids, and phenolics) are present [8,9]. However, to date, only a few studies have evaluated the potential of endophytic fungi inhabiting Sri Lankan biota [10,11].

Endophytes are known to live inside almost all plants and it is crucial to select the right plant to investigate endophytes for pharmacologically relevant bioactive compounds. As a result, plants that have been utilized for millennia as a complementary form of medicine are a significant source for endophytes for bioprospecting. Hence, in the current study *Geophila repens* (L.) I.M. Johnst (*G. repens*), a Sri Lankan medicinal plant implicated as an untapped source for endophytic fungi, was selected to identify endophytes. The genus *Geophila* (Rubiaceae) consists of 28

* Corresponding author at: Department of Pharmaceutical Biosciences, Uppsala University, BMC Box 591, Husargatan 3, 75124 Uppsala, Sweden.
E-mail address: sunithi.gunasekera@farmbio.uu.se (S. Gunasekera).

https://doi.org/10.1016/j.fitote.2023.105496
Received 9 January 2023; Received in revised form 24 March 2023; Accepted 25 March 2023
Available online 28 March 2023

species, of which *G. repens* is the most widespread [12]. It extends throughout the tropics from America to Africa, Madagascar, Asia, and Micronesia. Synonyms include *Rondeletia repens* L., *Geophila reniformis* D. Don and *Geophila herbacea* (Jacq.) O. Kuntze [13]. *G. repens* is used to make a decoction to treat tonsilitis, colds and coughs in traditional Sri Lankan medicine [14]. Several terpenes including β-caryophyllene, β-elemene, farnesyl butanoate, myrcene, and trans-nerolidol are found as major components in the essential oil of *G. repens*, which is reported to exhibit antibacterial activity against *P. aeruginosa* and *B. subtilis* [15]. Additionally, 1,2-phenylethyl 2,6-dihydroxybenzoate, a natural lipid lowering small molecule, has been isolated from the ethanolic extract together with benzyl 2-hydroxy-6-methoxybenzoate, the triterpene friedelin, and the furanocoumarin bergapten [16]. Aside from these, antioxidant and anticholinesterase activities of *G. repens*, presumably due to the presence of the phenolic compounds are also reported [17]. *G. repens* was one of the plants highlighted to contain specialized metabolites with antibacterial activities in its aqueous and organic extracts, during a bioactivity screening study we conducted on traditionally-used medicinal plants in Sri Lanka [18]. In a most recent follow-up study, we identified a series of cyclotides, stable cyclic peptides, with cytotoxicity against a Lymphoma cancer cell line and antibacterial activity against pathogenic bacteria, providing scientific evidence for its ethnomedical use. Transcriptomes of additional cyclotides and their putative biosynthetic enzymes were also identified, providing further support for the drug discovery potential of *G. repens* [19].

Until now, the endophytic microorganisms of *G. repens* have remained unstudied. In this study, eight fungal cultures were isolated from the leaves of *G. repens* and screened in a disc diffusion assay. The fungus *Xylaria feejeensis* showed strong antibacterial activity and was selected for further study. This led to the isolation of 6′,7′-didehydrointegric acid (**1**) and 13-carboxyintegric acid (**2**), two new derivatives of integric acid (**3**) [20], the key antibacterial constituent. Furthermore, the known compounds xyolide (**4**) [21], (4a*R*,5*S*)-3,4a-dimethyl-2-oxo-2,4,4a,5,6,7-hexahydronaphtho[2,3-*b*]furan-5-carboxylic acid (**5**) [22] and (*R*)-8-hydroxy-3-methyl-1-oxoisochromane-5-carbaldehyde (**6**) were also isolated in low quantities. Herein we describe the isolation, structure elucidation and antibacterial/cytotoxic testing of **1**–**6**.

2. Results and discussion

2.1. Identification of endophytic fungi

The usual practices in natural product discovery programs involve isolating microorganisms from samples, growing them at different temperatures in a variety of selective or nonselective media, and testing the extracts in a range of targeted screens for activity for potential industrial or pharmaceutical applications. The most encouraging and emerging development is investigating novel endophytes, with the expectation that unusual endophytes may produce previously undiscovered natural products. Hence, a previously established protocol was used [23] to identify the endophytic fungi from Sri Lankan medicinal plant *G. repens.* The sterilized *G. repens* plant parts were placed on Potato Dextrose Agar (PDA) plates at room temperature for 5 to 15 days. The endophytic fungi that emerged from the cut edges of the plant segments were repeatedly sub-cultured on antibiotic-free sterile PDA plates until pure cultures were obtained. Eight morphologically distinct endophytic fungi were identified by morphological examination under a light microscope (Fig. 1). A total of eight fungal strains, two *Colletotrichum* sp. and one strain each from *Phyllosticta* sp., *Daldinia* sp., *Trichophyton* sp., *Trichoderma* sp., *Aspergillus* sp. and *Xylaria* sp. were identified. All these fungi are commonly identified as endophytes in plants.

Molecular identification based on the ITS sequence data and the closest BLAST match were used to identify six of the fungal strains that showed antibacterial activity (Supplementary Table S1). The assembled DNA were searched for similar fungal sequences by the standard NCBI-blastn 2.4.0+ service. Three fungal isolates displayed 97% similarity and two displayed almost 100% similarity with the available data in NCBI GenBank. However, *Colletotrichum fructicola* displayed only 91% similarity to the GenBank data. The relevant accession numbers obtained from the NCBI GenBank for the submitted sequences are shown in Supplementary Table S1.

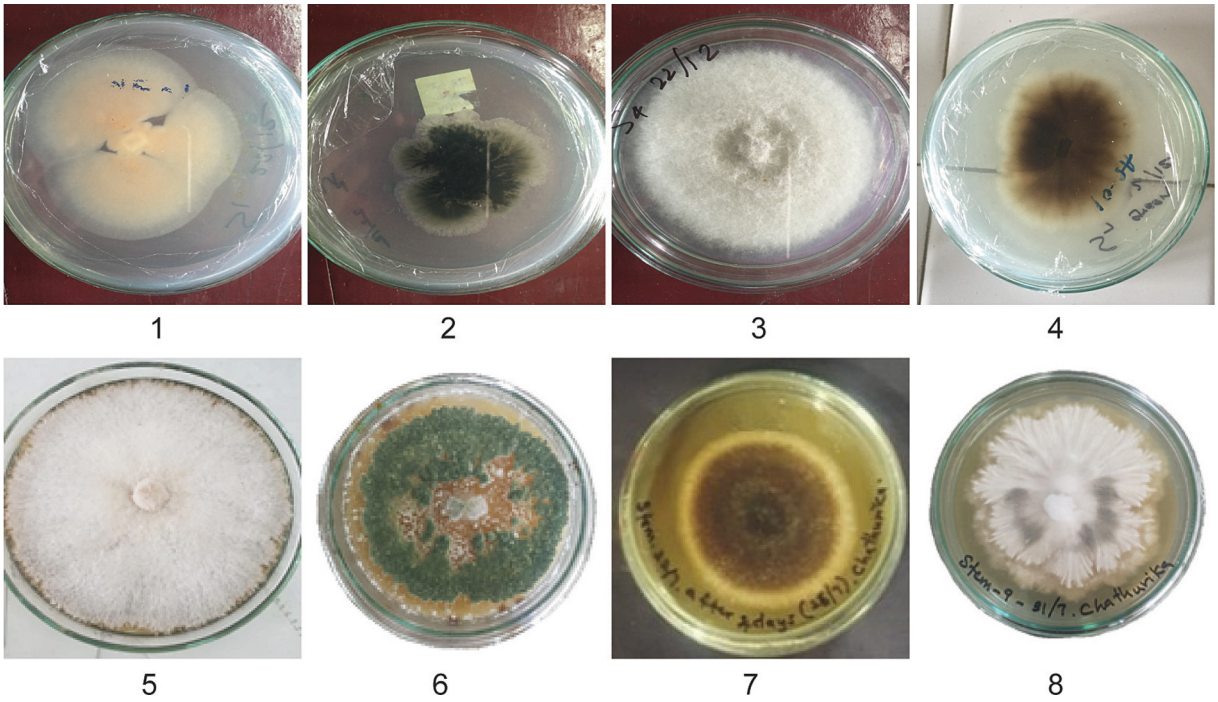

Fig. 1. Pure cultures of the endophytic fungi isolated from *Geophila repens. Colletotrichum gloeosporioides* (1), *Phyllosticta capitalensis* (2), *Colletotrichum fructicola* (3), *Daldinia eschscholtzii* (4), *Trichophyton* sp. (5), *Trichoderma* sp. (6), *Aspergillus niger* (7), *Xylaria feejeensis* (8). *Trichophyton* sp. and *Trichoderma* sp. were identified by morphological examination under a light microscope.

2.2. Antibacterial activity of the endophytic fungal crude extracts by disc diffusion assay

The antibacterial activity of endophytic fungal crude extracts at 500 μg/disk were obtained using an agar disc diffusion assay and the average inhibition zones were measured to the nearest millimetre [24]. The lipophilic and aqueous methanolic crude extracts prepared were tested against two Gram-positive (*S. aureus* and *B. cereus*) and two Gram-negative (*E. coli* and *P. aeruginosa*) bacterial strains (Table 1). Of the tested extracts, both the lipophilic and aqueous methanolic extracts of *Colletotrichum gloeosporioides* showed prominent antibacterial activity against both Gram-positive and Gram-negative bacteria. In particular, the extracts of *C. gloeosporioides* showed the highest antibacterial activity against *E. coli* (26.6 ± 0.8 mm) and *P. aeruginosa* (22.6 ± 1.2 mm). Furthermore, the lipophilic extract of *Phyllosticta capitalensis* and aqueous methanolic extract of *Aspergillus niger* also displayed broad spectrum of antibacterial activity against all tested Gram-positive and Gram-negative bacteria. However, the highest activity against *S. aureus* (27.3 ± 0.6 mm) and *B. cereus* (28.0 ± 0.1 mm) was exhibited by the crude lipophilic extract of *X. feejeensis* (Table 1). According to the clinical and laboratory standards institute (CLSI) standards, susceptible breakpoint is 20 mm or more with the normal dosage of an antimicrobial agent is considered as significant. Bioactivity that falls within the inhibition zone of 15–19 mm is considered as "intermediate antimicrobial susceptible" [25]. The results from agar disc diffusion assay was supportive that the antibacterial activity implicated in the crude *G. repens* extract [18] could be rationally linked to the metabolites produced by the endophytes. Endophytes work in harmony with their host and are known to produce unique specialized metabolites to assist the host effectively battle pathogens and pests [26].

2.2.1. Microfractionation and bioactivity screening of the lipophilic extract of Xylaria feejeensis

Among the tested endophytic fungi, *C. gloeosporioides*, *P. capitalensis*, *A. niger* and *X. feejeensis* stood out as the most likely sources of antibacterial compounds. As *X. feejeensis* showed the highest antibacterial activity for Gram-positive bacteria, it was selected for further isolation work. To identify antimicrobial constituents, 2 mg of the dried lipophilic extract of *X. feejeensis* was fractionated into 45 fractions by RP-HPLC. These fractions were then screened in a microdilution assay against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) [27]. While no growth inhibition against *E. coli* was observed, visual inhibition of *S. aureus* occurred in several fractions. This growth inhibition was maintained only up to nine hours in fractions 11–14 and 38–41, but notably no visible growth was observed in the fraction 29–36 even after 24 h against *S. aureus* (Supplementary Fig. S1). The bioactive wells were then analyzed by ESI-MS to establish the molecular masses associated with antibacterial constituents and guide their large-scale isolation (Supplementary Table S2).

Table 1
Antibacterial activity of endophytic fungi extracts isolated from *Geophila repens*.

Endophytic fungal strain	Extract type (500 μg/disc)	Average diameter of inhibition zone (mm)			
		E. coli	*P. aeruginosa*	*S. aureus*	*B. cereus*
Colletotrichum gloesporiodes	aqueous	26.6 ± 0.8[a]	22.6 ± 1.2	25.6 ± 1.8	20.3 ± 0.3
	lipophilic	22.0 ± 0.5	22.3 ± 1.4	20.3 ± 1.2	20.6 ± 0.3
Phyllosticta capitalensis	aqueous	20.3 ± 0.3	9.0 ± 1.5	IA	10.3 ± 0.3
	lipophilic	21 ± 1.5	13.3 ± 0.8	11.6 ± 0.8	10.3 ± 0.3
Colletotrichum fructicola	aqueous	12.6 ± 0.3	IA	IA	IA
	lipophilic	IA	12.3 ± 2.8	IA	IA
Daldinia eschscholtzii	aqueous	IA	IA	IA	IA
	lipophilic	IA	7.3 ± 0.3	11.5 ± 0.5	11.2 ± 0.5
Trichophyton sp	aqueous	IA	IA	IA	IA
	lipophilic	IA	IA	IA	IA
Trichoderma sp	aqueous	IA	IA	IA	IA
	lipophilic	IA	IA	8.3 ± 0.6	11 ± 0.1
Aspergillus niger	aqueous	17.1 ± 0	16.2 ± 0.1	16.5 ± 1.0	17.6 ± 0.1
	lipophilic	IA	IA	IA	IA
Xyleria feejeensis	aqueous	IA	IA	IA	IA
	lipophilic	IA	IA	27.3 ± 0.6	28.0 ± 0.1
Positive control	Gentamycin 10 μg/disc	8.3 ± 0.3	13.5 ± 0.5	10.3 ± 0.3	12.3 ± 0.3

[a] Standard error calculated using Minitab 17. IA = inactive.

2.3. Isolation of specialized metabolites from the lipophilic extract of Xylaria feejeensis

To begin, the dried lipophilic extract of *X. feejeensis* was fractionated into seven fractions (1–7) on a diol-bonded silica flash column by consecutive solvent mixtures of increasing polarity, employing hexane, EtOAc and MeOH (Supplementary Table S4). Each fraction was subjected to a microdilution assay to establish their corresponding MIC values [27]. Fraction 1 (100% hexane) was active against *S. aureus* at the highest tested concentration of 500 μg/mL, while fraction 2 (hexane/EtOAc (5:1), was active at 250 μg/mL. Fractions 3–6 showed activity at the highest tested concentration (500 μg/mL) against both bacterial species. No growth inhibition was observed for fraction 7.

Further fractionation of fractions 2–4 by reversed-phase chromatography led to the isolation of two new metabolites, 6′,7′-didehydrointegric acid (**1**) and 13-carboxyintegric acid (**2**) along with four known natural products, integric acid (**3**), xyolide (**4**), (4a*R*,5*S*)-3,4a-dimethyl-2-oxo-2,4,4a,5,6,7-hexahydronaphtho[2,3-*b*]furan-5-carboxylic acid (**5**) and (*R*)-8-hydroxy-3-methyl-1-oxoisochromane-5-carbaldehyde (**6**) (Fig. 2). Typically, the fractions from the medium pressure liquid chromatography (MPLC) were first analyzed by ^{1}H NMR and further purified by RP-HPLC, given a mixture of compounds were evident. Corresponding fractions from which the pure compounds were isolated are given in Supplementary Table S5. All compounds except **2** eluted between 75 and 85% CH_3CN in the gradient. Compound 2 eluted at ~65% CH_3CN concentration. The two new eremophilane sesquiterpenoids, 0.5 mg of (**1**) and 0.5 mg of (**2**) were isolated from fractions 3 and 4, respectively. Compound **3** was found as the major compound produced by *X. feejeensis*, occurring in large amounts in both fractions 3 and 4. Collectively, ~20 mg of **3** was isolated from the MPLC and purified RP-HPLC fractions. Presence of **3** was evident in other MPLC fractions as well, but these were not purified as the amount isolated was sufficient for bioassays. In total, an amount of 4.7 mg of **4** and 0.8 mg of **5** were isolated. Compound **6** appears to be a minor compound in the fungal extract as only a small amount could be isolated (0.2 mg).

2.4. Structural characterization of pure compounds

6′,7′-Didehydrointegric acid (**1**) was isolated as a yellow amorphous solid. (+)-HRESIMS analysis showed a molecular ion peak at *m/z* 429.2266 $[M + H]^+$, consistent with a molecular formula of $C_{25}H_{33}O_6^+$ (calcd 429.2272). The ^{1}H and HSQC spectra of **1** showed resonances associated with an aldehyde (δ_H 9.51, 1H), four olefinic methines (δ_H 6.60, 6.03, 5.36, 5.44, 4H), one methylidene (δ_H 6.45, 6.32, 2H), four aliphatic methines (δ_H 5.50, 3.76, 2.60, 2.45, 4H), four methylenes (δ_H 2.38–1.80, 8H) and four methyl groups (δ_H 1.84, 1.62, 1.48, 1.02, 12H) (Table 2). An additional seven non-protonated carbon resonances were observed in the ^{13}C NMR spectrum of **1**, corresponding to one sp^3 (δ_C 39.3) and six sp^2 hybridized carbons (δ_C 199.5, 176.1, 168.2, 162.0, 149.9, 127.5), of which the former three were predicted to be carbonyl groups. Comparison of the ^{13}C and ^{1}H NMR data of **1** to those of the co-isolate **3** indicated the two compounds to have similar structures [20].

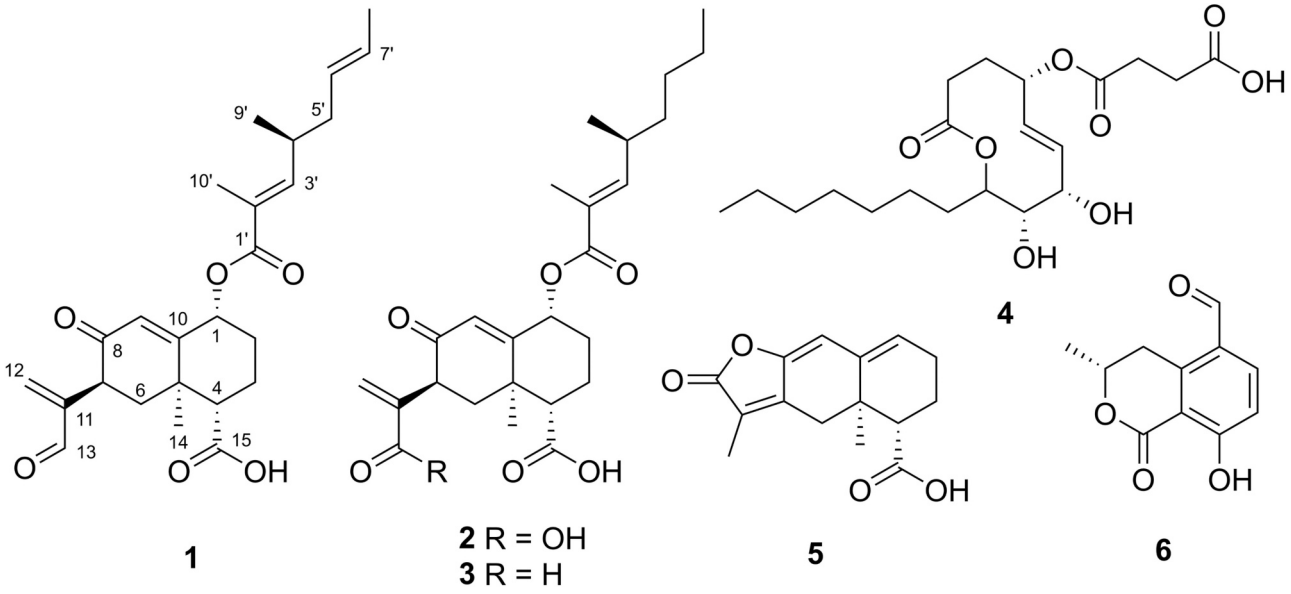

Fig. 2. Natural products isolated from *Xylaria feejeensis*. Dehydrointegric acid (**1**), carboxy integric acid (**2**), integric acid (**3**), xyolide (**4**), (4a*R*,5*S*)-3,4a-dimethyl-2-oxo-2,4,4a,5,6,7-hexahydronaphtho[2,3-*b*]furan-5-carboxylic acid (**5**) and (*R*)-8-hydroxy-3-methyl-1-oxoisochromane-5-carbaldehyde (**6**).

Three major differences in the NMR spectra of **1** and **3** were observed: the addition of two olefinic methine protons in **1** (δ_H 5.44, 5.36), the loss of two methylene groups at C-6′/C-7′, and the conversion of the methyl group at C-8′/H_3–8′ from a triplet in **3** (δ_H 0.87/δ_C 14.1) to a deshielded doublet of doublets (δ_H 1.62/δ_C 18.1) in **1**. This suggested an additional degree of unsaturation at C-6′/C-7′, which was confirmed by COSY correlations between olefinic resonances (H-6′/H-7′) to H_3–8′ and HMBC correlations from H_3–8′ to C-6′/C-7′ (Fig. 3). Slight deshielding of C-5′ (δ_C 40.7 in **1**; 36.5 in **3**) also supported this conclusion. The configuration of H-6′/H-7′ was determined to be *trans*- based on the observed coupling constant (15.2 Hz). With the planar structure of **1** established, the relative configuration of the four stereogenic centers in **1** were determined by comparison of NMR data to those of **3** and confirmed by the presence (H_3–14 to H-7) and absence (H_3–14 to H-1) of key ROESY correlations [20]. These data identified **1** as the new compound, 6′,7′-didehydrointegric acid.

13-Carboxyintegric acid (**2**) was isolated as a yellow amorphous solid. Analysis of its (+)-HRESIMS data revealed a molecular ion peak at *m/z* 447.2361 $[M + H]^+$, from which a molecular formula of $C_{25}H_{35}O_7^+$ was determined (calcd 447.2377). Comparison of the NMR data of **2** to **1/3** revealed a very similar structure. The key differences in the NMR data of **2** and **3** were the loss of the aldehyde group at C-13, the ~6–8 ppm shielding of C-11/C-12 (δ_C 147.7/136.5 in **3**; δ_C 141.2/128.3 in **2**), and the presence of an additional carboxyl group in **2** (δ_C 169.4). These data indicated the oxidation of the aldehyde at C-13 to a carboxylic acid, and this was supported by key $^3J_{CH}$ correlations from H-7/H_2–12 to C-13 (δ_C 169.4). As both compounds **1** and **2** showed only very weak optical rotation (possibly due to compound degradation and the only very small amounts isolated), we cannot offer a confident assignment of their absolute configurations from our acquired spectral data. However, based on their presumed biosynthetic origin from compound **3**, we predict that **1** and **2** have the same absolute configuration as **3**. In previous studies, several secondary metabolites including **3** and **4** have been isolated from *X. feejeensis* cultures obtained from host organisms of both plant or/and marine sponge origin [28,29] (Supplementary Table S3). Compound **5** was isolated and characterized from *Xylaria* sp. BL321 [22]. Although **6** was not identified in *Xylaria* sp. in previous studies, it has been isolated from other cultured fungi [30]. ^1H and ^{13}C NMR resonance assignments for **3–6** were in good agreement with published values [22,28–30].

2.5. Antibacterial and cytotoxic activities of the isolated eremophilane sesquiterpenoids

Compounds **1–3** were screened against *Bacillus subtilis* subsp. *Subtilis* 168 (ATCC 23857), Methicillin-Resistant *S. aureus* (MRSA) (ATCC 33591) by a microdilution assay employing a Muller-Hinton broth medium to obtain MIC values [31]. Compound **3** gave MIC values of 16 μg/mL for *B. subtilis*, and 64 μg/mL for MRSA. Notably, the two new derivatives of integric acid (**1** and **2)** and **4** did not show any inhibitory activity up to 64 μg/mL, the highest concentration tested. When screened for cytotoxicity using erythrocytes, **1–3** did not exhibit any hemolytic activity up to 45 μg/mL in final concentration. This indicates that **3** is selectively antibacterial at MIC concentrations against certain Gram-positive pathogens, with no undesirable hemolytic effects to red blood cells.

In a study carried out by Srisapoomi *et. al* in 2015, marginal antibacterial activities for **3** against *B. subtilis* (ATCC 6633) (MIC = 0.312 mg/mL), *S. saprophyticus* (ATCC 15305) (MIC = 0.833 mg/mL), *S. aureus* (ATCC, 25923) (MIC = 1.250 mg/mL), MRSA (DMST 20654) (MIC = 1.250 mg/mL), and *E. faecalis* (ATCC 29212) (MIC = 2.500 mg/mL) are reported [32]. Besides its antibacterial effects, **3** is a known HIV-1 integrase inhibitor with an IC_{50} of ~10 μM [20]. In our study, a much higher antibacterial activity was obtained, compared to the study by Srisapoomi et al. This discrepancy is likely due to the different bacterial strains used in the two studies.

Compounds **5** and **6** were not screened in antibacterial assays as they were isolated in insufficient quantities. Notably, **4** is reported to exhibit low antibacterial activity with a MIC of 425 μM against an oomycete plant pathogen *Pythium ultimum* [21]. No antibacterial effects are found in literature for **5** and **6**, but **5** isolated from *Xylaria* sp. BL321 collected in South China is reported to have no cytotoxicity up to 50 μM against human breast cancer cell line MCF-7 [22].

Although the current study focused on a single endophytic fungal species, we have highlighted the potential of *G. repens* as a rich source of endophytic fungi. In the disc diffusion assay, the isolated fungi *C. gloeosporioides* also showed antibacterial effects. *C. gloeosporioides* is a plant pathogen that has been the source of compounds showing antifungal [33], antibacterial [34,35], anticancer [36] and anti-obesity [37] properties. Specifically, the antimicrobial tridepside colletotric acid is reported from *C. gloeosporioides* [35]. Additional secondary metabolites such as the piperidine alkaloid piperine has been characterized from

Table 2
NMR Spectroscopic Data for 6′,7′-didehydrointegric acid (**1**) and 13-Carboxyintegric acid (**2**) in CD_3OD.

Position	6′,7′-didehydrointegric acid (**1**)			13-carboxyintegric acid (**2**)	
	δ_c[a], type	δ_H[b] (*J* in Hz)	HMBC[c]	δ_c[a], type	δ_H[b] (*J* in Hz)
1	74.7, CH	5.50, dd (3.0, 3.0)	1′, 3, 5, 9, 10	74.7, CH	5.50, dd (3.0, 3.0)
2	30.9, CH_2	*α* 1.83, m *β* 2.12, m	1, 3, 4, 10 1, 3	30.8, CH_2	*α* 1.82, m *β* 2.11, m
3	21.4, CH_2	*α* 2.31, m *β* 1.80, m	2, 4, 5 1, 5, 15	21.4, CH_2	*α* 2.29, m *β* 1.80, m
4	54.8, CH	2.45, dd (12.7, 2.7)	2, 3, 5, 6, 14, 15	54.8, CH	2.46, dd (13.2, 2.3)
5	39.3, C	–	–	39.3, C	–
6	44.5, CH_2	*α* 2.38 dd (13.8, 13.8) *β* 2.10, m	4, 5, 7, 8, 11, 14 4, 5, 7, 8, 10, 14	45.1, CH_2	*α* 2.48, m *β* 2.14, m
7	44.9, CH	3.76, dd (14.4, 4.3)	5, 6, 8, 11, 12, 13	48.0, CH	3.68, dd (14.2, 4.2)
8	199.5, C	–	–	200.3, C	–
9	130.3, CH	6.03, s	1, 5, 7, 10, 14	130.3, CH	6.02, s
10	162.0, C	–	–	161.9, C	–
11	149.9, C	–	–	141.2, C	–
12	137.7, CH_2	6.45, s 6.32, s	7, 8, 11, 13 7, 8, 11, 13	128.3, CH_2	6.33, s 5.72, s
13	194.9, C	9.51, s	–	169.4, C	–
14	20.4, CH_3	1.48, s	4, 5, 6, 10	20.5, CH_3	1.48, s
15	176.1, C	–	–	176.3, C	–
1′	168.2, C	–	–	168.3, C	–
2′	127.5, C	–	–	127.4, C	–
3′	149.8, CH	6.60, dq (9.9, 1.6)	1′, 2′, 4′, 5′, 9′, 10′	150.6, CH	6.58, dq (10.0, 1.4)
4′	34.8, CH	2.60, m	2′, 3′, 5′, 6′, 9′	34.4, CH	2.56, m
5′	40.7, CH_2	2.09, m 2.03, m	3′, 4′, 6′, 7′, 9′ 3′, 4′, 6′, 7′, 9′	37.7, CH_2	1.43, m 1.32, m
6′	129.6, CH	5.36, m	5′, 7′, 8′	30.9, CH_2	1.24, m
7′	128.1, CH	5.44, dd (15.2, 6.3)	5′, 6′, 8′	23.8, CH_2	1.30, m
8′	18.1, CH_3	1.62, dd (6.3, 1.4)	6′, 7′	14.4, CH_3	0.89, t (7.0)
9′	19.7, CH_3	1.02, d (6.7)	3′, 4′, 5′	20.3, CH_3	1.01, d (6.5)
10′	12.8, CH_3	1.84, d (1.6)	1′, 2′, 3′	12.8, CH_3	1.85, s

[a] 150 MHz.
[b] 600 MHz.
[c] HMBC correlations are from proton(s) stated to the indicated carbon.

C. gloeosporioides of *Piper nigrum* [38]. The other fungi isolated herein, *Phyllosticta capitalensis* and *A. niger*, are widespread endophytic fungi [39–41].

3. Conclusions

In the current work, eight endophytic fungi species were isolated from *G. repens*, of which *X. feejeensis* was identified to produce specialized metabolites active against *B. cereus* and *S. aureus*. By further fractionation of the extract, the antibacterial activity was primarily delineated to **3**. This supports the notion that secondary metabolites produced by endophytic fungi of *G. repens* may be a contributing factor for the antibacterial activity implicated for *G. repens* in traditional Sri Lankan medicine. Although two structurally similar compounds (**1** and **2**) were also obtained, these did not show activity at the highest tested concentration of 64 μg/mL.

This study has raised awareness on the importance of exploring new biotechnological applications for endophytic fungi as a source of new bioactive secondary metabolites. The established protocol of growing fungi under laboratory conditions via surface-sterilized plant parts, without any damaging effects to the host plant applied herein, allowed bioactive fungal metabolites to be obtained in a sustainable manner. In future studies, by subjecting the isolated compounds to a wide array of additional bioassays, new bioactivities could be potentially established for the isolated compounds that could be of therapeutic relevance. Additionally, our study has paved the path for future investigation of fungi biosynthetic pathways of *G. repens* via methods including genome mining to establish the potential of specialized metabolites and their derivatives as antimicrobial agents.

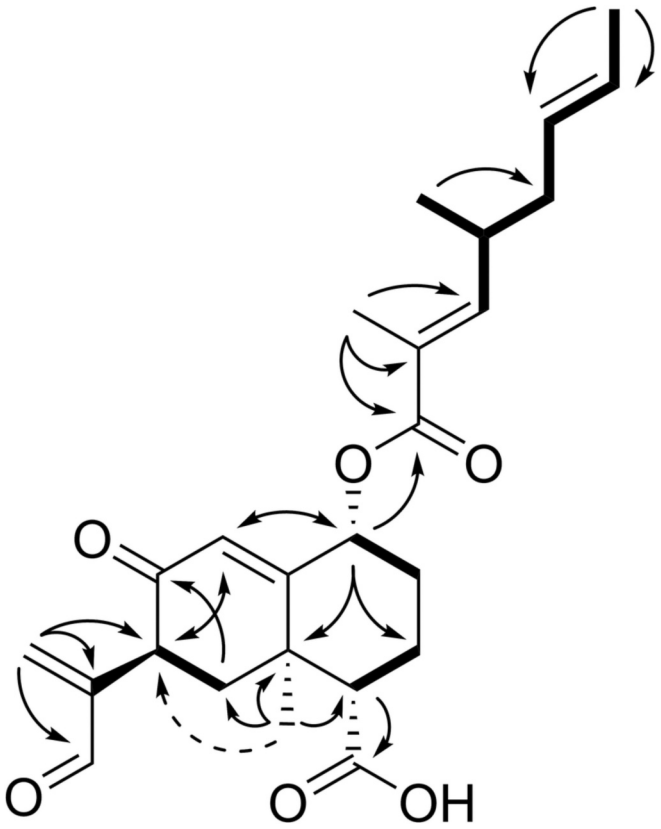

Fig. 3. Key COSY (bold bonds) and HMBC (solid arrows) and ROESY (dashed arrow) correlations in **1**.

4. Experimental

4.1. General experimental procedures

Mass analysis of microfractionated wells and MPLC/HPLC fractions was carried out on a Waters nanoAcquity ultra performance liquid chromatography (UPLC) system coupled to a Micromass Q-TOF micro mass spectrometer (MS) operated in positive mode. Final pure compounds were analyzed on Xevo G2-XS quadrupole time-of-flight (QToF) mass spectrometer coupled with a nano-Acquity UPLC (Waters Corp. Milford, MA, USA) operated in positive mode. NMR spectra were acquired on a Bruker Avance Neo 600 MHz (TCI (CRPHe TR-1H and 19F/13C/15 N 5 mm-EZ)) spectrometer at 298 K. NMR data were acquired for all compounds after dissolving in methanol-d_4. Optical rotations were recorded on a PerkinElmer 241 polarimeter (PerkinElmer, Waltham, MA, USA), and $[\alpha]_D$ values are given in 10^{-1} deg. cm^2 g^{-1}.

Analytical grade solvents, MeOH, hexane, EtOH, EtOAc, NaOCl and CH_3CN were obtained from either Sigma-Aldrich, UK or VWR (Sweden). Formic acid (FA) and trifluoroacetic acid (TFA) purchased from VWR

(Sweden). Tris base (Sigma-Aldrich) used to prepare tris buffer of pH 7.8. Potato Dextrose Agar (PDA, HIMEDIA) used for fungal cultivation. Microfractionated extracts were collected in 96 deep well plates (VWR, Sweden). Polystyrene U-bottom plates (Thermo Fisher Scientific) were used for the microdilution and cytotoxicity assay. A savant Speed Vac plus SC110A centrifugal evaporator, and BUCHI-R-200 rotary evaporator were used to evaporate the solvent in microtiter plates and round bottom flasks respectively.

4.2. Plant material

The leaves of *Geophila repens* (L.) I.M. Johnst were collected from Navinna ayurvedic garden in Navinna (6°50′58″N 79°55′25″E) in Colombo District, Sri Lanka. The plants were authenticated by comparison with the voucher specimen stored at the National Herbarium at Royal Botanical Gardens, Peradeniya, Sri Lanka. The plant specimens were identified at the National Herbarium at the Royal Botanical Garden, Peradeniya by N.P.T. Gunawardena. A voucher specimen (No. UOC/NPSR/010) of *G. repens* was deposited in the herbarium of Department of Plant Sciences, University of Colombo, Sri Lanka.

4.3. Isolation and identification of endophytic fungi from Geophila repens (L.) I.M. Johnst

The authenticated plant specimens were brought to the laboratory in sealed polythene bags and the isolation of endophytic fungi was carried out within five hours of collection using a previously established method [23]. Healthy leaves, roots, stems, and flowers of the plant were surface sterilized by sequentially washing with sterilized distilled water (2 min), 70% EtOH (30 s), 5% NaOCl (1.5 min) and again sterilized water (1 min). Then, sterilized plant parts (leaves, stem, root, and flower segments) were blot-dried with sterilized filter paper, cut into 0.5 cm^2 segments using a sterilized sharp blade and placed on Potato Dextrose Agar (PDA) enriched with the antibiotic (ciprofloxacin 150 mg/L) under aseptic conditions at room temperature (29 ± 2 °C) for 5 to 15 days. The endophytic fungi that emerged from the cut edges of the plant segments were repeatedly sub-cultured on antibiotic-free sterile PDA until pure cultures were obtained. PDA culture plates without plant parts were also incubated under same conditions as a control.

The isolated pure endophytic fungi were initially identified by colony morphological features through microscopic examination of hyphae and reproductive structures. The identities were further confirmed using standard molecular biological techniques. Fungal DNA was extracted from 15 mg of mycelia using a published protocol [23]. The target ITS region including the 5.8S gene was amplified by polymerase chain reaction (PCR) using universal ITS 1 and ITS 4 [42] under thermal cyclic conditions. Initial denaturation of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, annealing at 1 min for 55 °C and 2 min at 72 °C, with a final extension of 7 min at 72 °C [23,43]. Amplified DNA was sequenced and analyzed by BLAST software [National Center for Biotechnology Information (NCBI)] and accession numbers were obtained for the gene sequences.

4.4. Large scale culturing of endophytic fungi to obtain crude fungal extracts

Each isolated endophytic fungus was grown separately on PDA plates and incubated for two to three weeks. When the fungi reached the sporulation stage, each fungus together with the culture medium was cut into small pieces and immersed in 200 mL of EtOAc/MeOH (9:1) overnight. The resulting extracts were filtered, and the filtrates were evaporated to dryness under reduced pressure by rotary evaporator at 40 °C. The residual fungal material was extracted again using 200 mL of MeOH/H_2O (3:2) overnight. The resulting aqueous methanolic (MeOH/H_2O) extracts were filtered and the MeOH was removed using a rotary evaporator. The remaining water fraction was freeze dried to obtain dried aqueous crude fungal extracts.

4.5. Antibacterial activity using agar disc diffusion assay

The EtOAc and aqueous methanolic extracts of fungi were tested in triplicate against four pathogenic bacteria *Escherichia coli* (ATCC 35218), *Psuedomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 25928), *Bacillus cereus* (ATCC 11718) at 500, 250, 125 and 62.5 μg/disc concentrations using standard disc diffusion assay [24,44]. Twenty μL of EtOAc or MeOH was added as negative control and gentamycin (Sigma Aldrich, USA) was used as a positive control (10 μg/disc).

4.6. Microfractionation of ethyl acetate extract

The dried ethyl acetate extract (2 mg) was re-suspended in 20 μL of 60% CH_3CN and diluted up to 10% CH_3CN by the addition of 180 μL of Milli-Q water. The volume of 200 μL was then injected on the column and was subject to microfractionation using Shimadzu LC-10 HPLC system equipped with a SPD-M10AVP Photodiode Array Detector (PDA). A Phenomenex Jupiter C_{18} (250 × 4.6 mm, 5 μm, 300 Å) column with a gradient from H_2O/0.05% TFA (95%)/CH_3CN/0.05% TFA (5%) to H_2O/0.05% TFA (3%)/CH_3CN/0.05% TFA (97%) was employed at a flow rate of 1 mL/min. Fourty-five fractions were collected at one-minute intervals to a 96 deep well plate over 45 min. One hundred μL of each fraction was transferred to an untreated, polystyrene U-bottom plate and dried using a centrifugal evaporator (Savant Speed Vac plus).

4.7. Isolation of compounds

Five grams of EtOAc extract was dissolved in MeOH and dry loaded onto eight grams of silica gel using a rotary evaporator. The extract-impregnated gel was then loaded into a precolumn and connected in series to a diol-bonded silica flash column (Interchim PF-DIOL, 30 μm, 40 g). By employing a stepwise elution program using hexane, EtOAc and MeOH (each solvent/solvent mixture for 16 min at 10 mL/min), seven fractions were collected: fraction 1: hexane (100%), fraction 2: hexane/EtOAc (5:1), fraction 3: hexane/EtOAc (1:1), fraction 4: EtOAc/hexane (7:3), fraction 5: (EtOAc 100%), fraction 6: EtOAc/MeOH (7:3), fraction 7: MeOH (100%). Each fraction was then dried under vacuum. Fraction 2 was directly purified at 3 mL/min on a semi-preparative RP-HPLC column (250 × 10 mm, 5 μm, Phenomenex C_{18}) by a gradient of 5–100% CH_3CN/0.05% TFA in 60 min. For large scale isolation, the total dry residue from fractions 3 and 4 (~0.48 g and ~ 0.8 g respectively) were separately mixed with C_{18} gel (1:1 *w*/w) and fractionated using reversed-phase MPLC (Äkta FPLC from Amersham Pharmacia Biotech, GE Healthcare) by a H_2O/0.05% TFA and CH_3CN/0.05% TFA linear gradient of 0–100% CH_3CN/0.05% TFA over 60 min (Biotage Sfär C_{18} Duo 100 Å 30 μm 12 g, 10 mL/min). The fractions collected from MPLC that contained a mixture of compounds as evident by NMR, were further purified using a Shimadzu LC-20 by semi-preparative RP-HPLC (250 × 10 mm, 5 μm, Phenomenex C_{18}). Each MPLC fraction was first dissolved in 300 μL of 100% DMSO and injected to the RP-HPLC column followed by a H_2O/0.05% TFA and CH_3CN/0.05% TFA gradients developed according to the polarity of the compounds present. Compounds **1** and **3** were purified using a RP-HPLC gradient of 5–50% CH_3CN/0.05% TFA for the first 25 min and 50–90% CH_3CN/0.05% TFA over the next 35 min. Compound **5** was purified using a RP-HPLC gradient of 5–100% CH_3CN/0.05% TFA over 60 min. Compounds **2** and **4** were obtained directly as pure compounds, from the MPLC fractions and did not require further semi-preparative RP-HPLC purification. Details of the MPLC fractions from which the compounds were isolated are given in Supplementary Table S5.

4.8. Microdilution assay

A two-step microdilution assay [27] employing *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) was conducted to determine the antibacterial activity of the microfractionated ethyl acetate extract and fractions 1–7 from diol fractionation in triplicate. The microbes were obtained from the Department of Clinical Bacteriology at Lund University Hospital. In brief, the bacterial cell suspension was diluted in tris buffer (10 mM, pH 7.8 at RT) to 100,000 CFU/mL (measured at OD600). Dried U bottom plates were prepared by transferring 100 μL from each fraction. Fifty μL of Tris buffer and 50 μL of bacterial suspension were added to the plates prior to incubation. A stock concentration of 2 mg/mL was prepared from fractions 1–7 by dissolving in either Milli-Q water or 20% DMSO depending on the solubility. Fifty μL of the stock solution and 50 μL of Tris buffer were transferred to the corresponding first well. The other wells were filled only with 50 μL of Tris buffer. Two-fold serial dilution was carried out within the concentration range from 3.9 to 500 μg/mL followed by addition of 50 μL of bacterial suspension. 2 mg/mL of ciprofloxacin (Sigma Aldrich, USA) and 100 μL of Tris buffer alone (without the extract or bacterial cell suspension) were used as the positive and negative controls respectively.

The isolated pure compounds **1–3** were tested against *B. subtilis* subsp. *Subtilis* 168 (ATCC 23857), methicillin-resistant *S. aureus* (ATCC33591) to determine their MIC values [31]. Broth microdilutions for MIC tests were performed in microtiter 96-well plates with two-fold dilutions in ten consecutive wells in biological duplicates suspended in MH media. A volume of 50 μl bacterial solution (turbidity measured at OD600), were added in each well. Starting concentration of compounds were 64 μg/mL. Interpretation was performed after 16–20 h of incubation at 37 °C. Method precision was set at ±1 two-fold concentration. The lowest compound concentration with no visual growth represented the MIC.

4.9. Cytotoxicity assay

To evaluate the in vitro hemolytic potential of compounds **1–3**, cytotoxicity assay was used by measuring the hemolytic activity freeing the hemoglobin contained in erythrocytes as previously described [45]. Compounds **1–3** (45 μg/mL in final assay) were incubated with fresh heparinized blood (Uppsala University Hospital). After incubation of microtiter plates at 37 °C for 45 min with shaking (250 RPM), red blood cells are pelleted by centrifugation at 1000 *xg* for 10 min at room temperature. The hemoglobin concentration remaining in plasma is measured spectrophotometrically at 540 nm.

Author statement

Sanjeevan Rajendran, Chamari Hettiarachchi and **Sunithi Gunasekera:** conceptualized and designed the study, **Sanjeevan Rajendran** and **Chathurika Fernando:** plant collection, isolation of endophytic fungi and preparation of endophytic extracts, **Sunithi Gunasekera:** isolation of antibacterial compounds. **Sanjeevan Rajendran** and **Lakmini Kosgahakumbura:** antibacterial screening of the crude endophytic extracts, **Luke P. Robertson:** acquired and analyzed NMR data, **Ulf Göransson:** setting-up the microfractionation and chromatography-based compound isolation protocols, **Helen Wang:** antibacterial assays on pure compounds, **Sanjeevan Rajendran, Chamari Hettiarachchi, Luke P. Robertson** and **Sunithi Gunasekera:** writing, reviewing and editing with the input from other authors.

Declaration of Competing Interest

Authors declare that they no conflict of interest for the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by Swedish Research Council's Linkage grant (2013-06672) awarded to SG. We would like to acknowledge NMR Uppsala infrastructure, which is funded by the Department of Chemistry - BMC and the Disciplinary domain of Medicine, Uppsala University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2023.105496.

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