Antibacterial metabolites from the Sri Lankan demosponge-derived fungus, *Aspergillus flavipes*

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Sri Lankan sponge-derived fungi are an unexploited resource awaiting exploration for drug leads. Three fungi from the internal tissues of a demosponge collected on the west coast of Sri Lanka were isolated and antimicrobial activities of their crude extracts were evaluated. Aspochalasin B, D, M, asperphenamate and 4-OMe asperphenamate were isolated from the crude ethyl acetate extract of the fungus *Aspergillus flavipes*. Aspochalasin B and D showed antibacterial activities against *Bacillus subtilis* (MIC, 16 and 32 μg ml^{-1} respectively), *Staphylococcus aureus* and MRSA (MIC, 32 μg ml^{-1}). The results highlight the potential of isolating bioactive secondary metabolites from Sri Lankan sponge-derived fungi.

Keywords: *Aspergillus flavipes*, aspochalasin, asperphenamate, demosponge, marine.

OCEANS are home to a variety of lifeforms that are potential sources for novel drug discovery. Comparatively, marine organisms have a shorter history than the long-standing history of terrestrial organisms, for utilization in the field of medicine[1]. Sessile marine organisms such as sponges are known to rely mainly on toxic or noxious secondary metabolites for defence against predators and pathogens, and during allelopathic competition with neighbouring organisms for space or resources[2]. Consequently, marine invertebrates have become established as an outstanding producer of biological molecules with unprecedented structural features and important bioactivities[3]. However, many such compounds originally regarded as products of marine invertebrates have subsequently turned out to be biosynthesized by host associated microorganisms. A convincing example is the secondary metabolite jasplakinolide which initially was presumed to be produced by a *Jaspis* sp. (Phylum: Porifera), but turned out to be actually biosynthesized by a sponge-derived fungus[4].

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The limited number of recent studies on fungi derived from marine organisms has been productive and yielded many novel bioactive natural products. With the number of compounds reported increasing annually, there is great potential to isolate novel bioactive metabolites from these sparsely studied microorganisms[5]. Sponges have been shown to yield the greatest taxonomic diversity for marine-derived fungi and not surprisingly fungi isolated from marine sponges account for the discovery of the majority of novel fungal metabolite from the oceans[5,6].

Marine molluscs, sponges and soft corals of Sri Lanka, an island surround by the Indian Ocean, have yielded numerous novel metabolites[7–10]. However, fungi associated with Sri Lankan marine organisms, remains an untapped resource that awaits exploration. We report here the isolation of fungi from the internal tissues of a marine demosponge, the antimicrobial activities of the crude extracts of the fungi, and the bioassay guided isolation of active metabolites from the crude extract of *Aspergillus flavipes* (KR063133), along with structure elucidation and antimicrobial activities of the isolated compounds.

Materials and methods

Isolation of fungi from a demosponge

A marine sponge of the class Demospongiae was collected from the Indian Ocean, near Dehiwala, Sri Lanka, in January 2014 and brought to the laboratory cooled in ice. Fungal isolation from the internal tissues was carried out within 12 h of sponge collection. Sponge voucher specimen (PR-D4) was deposited at the Natural Products Laboratory, Department of Chemistry, University of Colombo. To isolate the fungi occupying the internal tissues, the sponge was washed well with sea water and a small section was cut and was surface sterilized with 70% ethanol for 1 min. Then, this was cut into small pieces under sterile conditions and was crushed using a small

amount of sterilized distilled water. The extract obtained was spread on pre-prepared sterile media dishes of potato dextrose agar (PDA), starch yeast peptone agar (SYP), yeast peptone dextrose agar (YPD), malt agar (ME) and malt peptone dextrose agar (MEA), enriched with antibiotic (chloramphenicol 50 mg/l) under aseptic conditions, and incubated at room temperature for several days. The media were prepared using unpolluted sea water in order to provide a marine environment for the normal growth of fungi. The fungi appearing on the media were transferred on to new PDA medium dishes prepared using sea water and sequence subculturing was done until pure cultures were obtained.

Antimicrobial evaluations of the crude fungal extracts

Each isolated fungus was grown on five PDA dishes and after the incubation period, fungus plus the medium were cut, immersed in 200 ml of ethyl acetate (EtOAc) for 24 h, filtered and the filtrate was evaporated to dryness using a rotary evaporate (BUCHI-R-200). The resulting crude fungal extracts were tested in triplicate against three Gram positive bacteria, *Staphylococcus aureus* (ATCC 43300), methicillin resistant *Staphylococcus aureus* (MRSA, ATCC 33591), *Bacillus cereus* (UBC 344), two Gram negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (UBC 8161) and a pathogenic fungus *Candida albicans* (ATCC 90028) at 200 μg/disc using the standard agar disc diffusion assay[11]. [+ve controls - Polymyxin B (30 μg/disc) for *B. subtilis*, *P. aeruginosa* and *E. coli*, Rifamycin (10 μg/disc) for *S. aureus* and MRSA and Amphotericin B (20 μg/disc) for *C. albicans*. Methanol solutions were used to transfer the crude extracts on to the discs and discs were dried prior to testing; –ve control – methanol]. The mean diameter of the zones of inhibition was recorded after overnight incubation.

Identification of the isolated bioactive fungus

The bioactive fungus was identified using morphological features and molecular biological techniques. For molecular identification, the fungal genomic DNA was extracted using a published protocol while the PCR and DNA sequencing were done commercially[12]. The acquired gene sequence was analysed by BLAST to obtain the identity of the fungus. The DNA sequence was also submitted to the NCBI GenBank database and an accession number was obtained. The preserved fungal voucher specimens were stored at 4°C at the Natural Products Laboratory, Department of Chemistry, University of Colombo, Sri Lanka.

Extraction and isolation of the bioactive secondary metabolites

The bioactive fungal strain (PR-D4-3) was grown on sterile PDA in 300 petri dishes (size, 100 × 20 mm) prepared using sea water, and incubated for 10 days at room temperature. Next, the fungus and culture medium were cut into small pieces and were extracted with EtOAc (3 × 1 l). The extracts were filtered and the combined extract was concentrated *in vacuo* and was tested for antimicrobial activity against the three Gram-positive bacteria, MRSA, *S. aureus* and *Bacillus subtilis* at 50 μg/disc using the agar disc diffusion method to confirm the activity[11].

To isolate the bioactive components from the complex mixture of the EtOAc extract, a series of bioassay guided chromatographic techniques that included in sequence, size-exclusion chromatography on Sephadex LH-20, normal phase silica gel chromatography using gradient elution and final reversed phase high performance liquid chromatography (HPLC) purifications were performed. The details of the isolation protocol which resulted in the isolation of five pure compounds **1**–**5**, are given in Figure 1.

Structure elucidation of the isolated compounds

The structure elucidation of the isolated compounds **1**–**5** was accomplished by analysis of NMR and mass spectral data. ^{1}H, ^{13}C and 2D NMR spectral data sets for **1**, **3** and **4** in DMSO-d_6 were obtained using a Bruker AV-600 spectrometer with a 5 mm CPTCI cryoprobe. Low resolution ESI-QIT-MS were recorded on a Bruker-Hewlett Packard 1100 Esquire–LC system mass spectrometer. The structures of the compounds **2** and **5** were obtained by comparison of ^{1}H and ^{13}C data (in DMSO-d_6) with previously reported data (**2** in $CDCl_3$ and **5** in DMSO-d_6).

Antimicrobial activities of the isolated compounds

Compounds **1**–**5** were tested for antimicrobial activity against three Gram-positive bacteria, *B. subtilis* (UBC 344), *S. aureus* (ATCC 43300) and methicillin resistant *S. aureus* (MRSA, ATCC 33591), two Gram-negative bacteria, *E. coli* (UBC 8161), *P. aeruginosa* (ATCC 27853) and pathogenic fungus *C. albicans* (ATCC 90028). The broth micro-dilution method according to the National Committee for Clinical Laboratory Standards with replacement using Mueller Hinton broth as the medium was used to determine the minimum inhibitory concentrations (MICs) of the compounds[13]. The MIC end point was taken as the lowest concentration with more than 90% growth inhibition. ADTX 880 (Beckman Coulter Inc.) plate reader was used to determine the optical density of the microbial growth at 600 nm. The positive

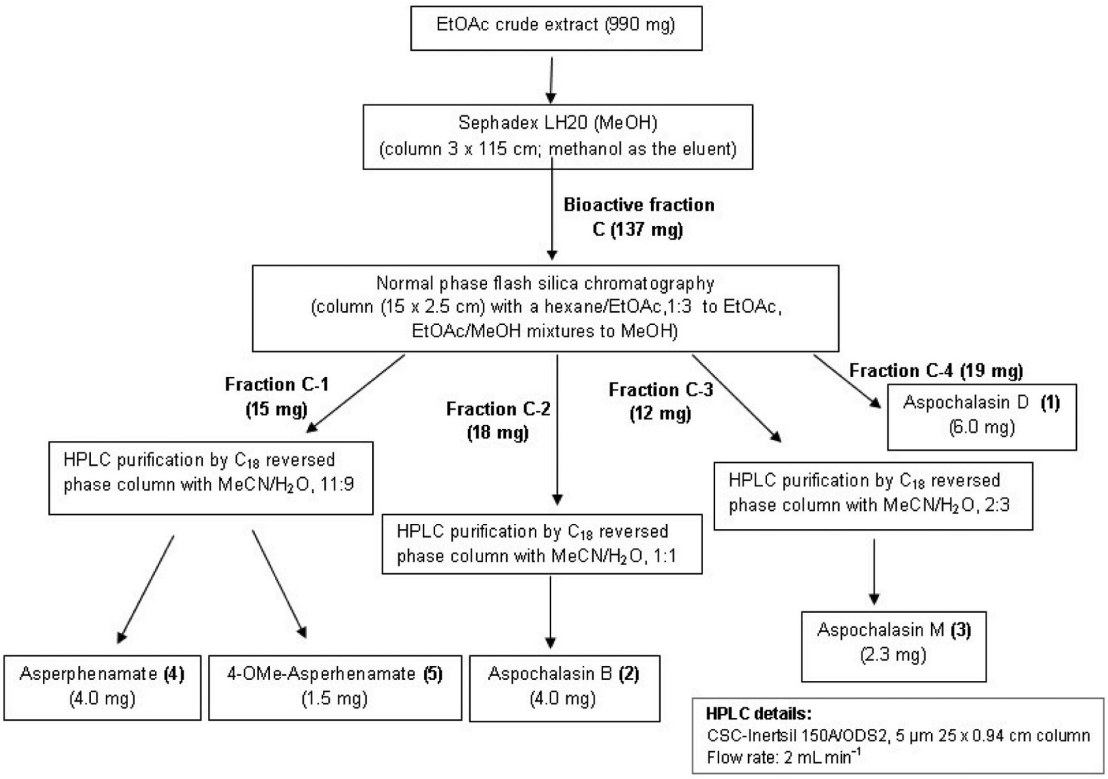

Figure 1. Schematic diagram for the compounds isolation protocol.

controls used were commercially available polymyxin B for *B. subtilis*, *E. coli* and *P. aeruginosa*, rifamycin for *S. aureus* and MRSA and amphotericin for *C. albicans*.

Results

Isolation, antimicrobial activity of the crude extracts and identification of the fungi

Three fungi were isolated from the surface sterilized tissues of the demosponge using media rich in malt. Fungi PR-D4-1 and PR-D4-3 were isolated from ME medium, whereas PR-D4-2 fungus was isolated from MEA medium. The crude EtOAc extract of the fungus PR-D4-3, showed antibacterial activity against the Gram positive bacteria *B. subtilis*, MRSA and *S. aureus* with zones of inhibition 15, 17 and 17 mm, respectively for the initial screening at 200 µg per disc. The crude extract of PR-D4-3 showed no activity for Gram-negative *E. coli* or *P. aeruginosa* and the pathogenic fungus *C. albicans*. The extracts of the other two isolated fungi, PR-D4-1 and PR-D4-2, were inactive for all the tested microorganisms.

Fungal mycelium of PR-D4-3 fungus was white and secreted a yellow pigment to the medium within 6–7 days of culture. According to the DNA sequence and blast results, this fungus showed 100% identity to the previously isolated *Aspergillus flavipes* species (e.g. accession number AY373849). Therefore, on the basis of its internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, the PR-D4-3 fungus isolated in the current study was assigned as *A. flavipes* (NCBI GenBank accession number: KR063133).

Isolation of the secondary metabolites

From 300 fungal cultured petri dishes, 1.6 g of crude EtOAc extract was obtained. The bioassay guided fractionation of 990 mg of crude EtOAc extract PR-D4-3 led to the active fraction C (137 mg) after the Sephadex LH20 column chromatography. Normal phase flash silica chromatography led to 15, 18, 12 and 19 mg of fractions C1-4 respectively. Fraction C4, a white powder, consisted of aspochalasin D (6 mg, **1**), which showed an R_f value of 0.56 on normal phase TLC with MeOH/EtOAc, 1 : 19. Reversed phase HPLC purification of fractions C2 and C3 led to 4.0 and 2.3 mg of aspochalasin B (**2**) and M (**3**) respectively, as white powders. Asperphenamate (**4**) (4 mg) and 4-OMe-asperphenamate (**5**) (1.5 mg) (Figures 2 and 3) were obtained as a white residue after HPLC purification of fraction C1. The HPLC retention times for

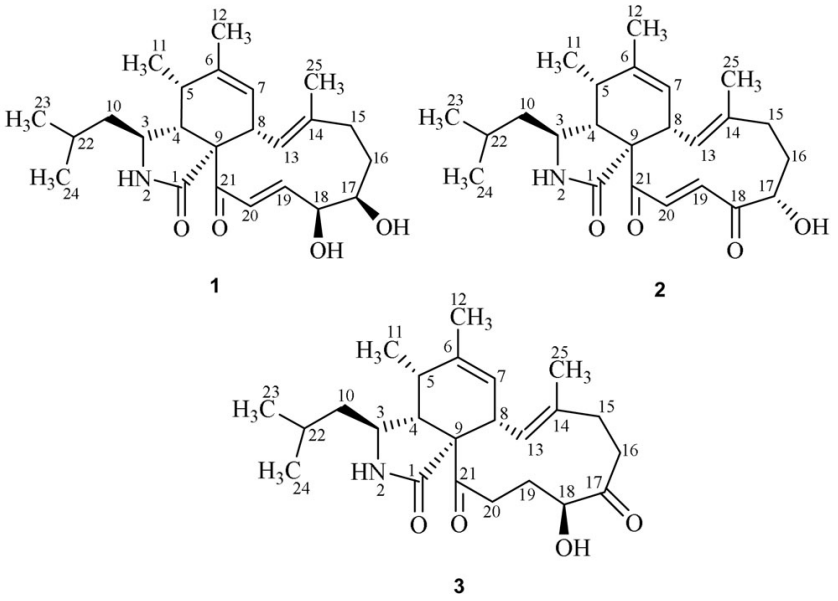

Figure 2. Chemical structures of aspochalasin D (**1**), B (**2**) and M (**3**).

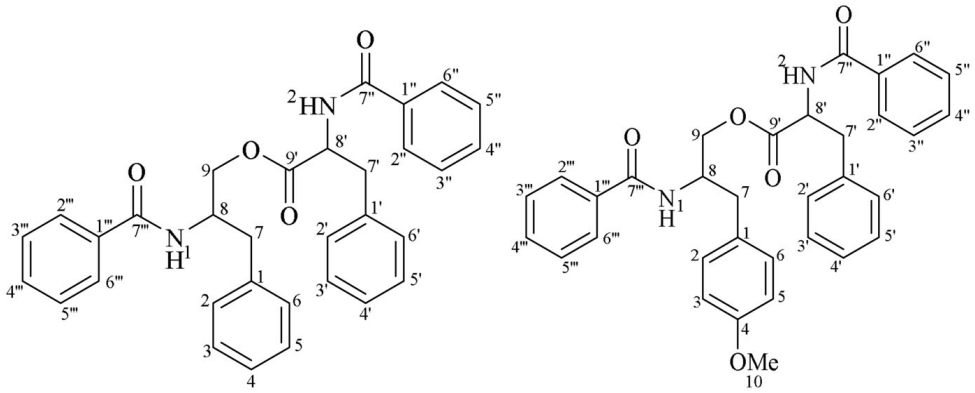

Figure 3. Chemical structures of asperphenamate (**4**) and 4-OMe-asperphenamate (**5**).

compounds **1**–**5** were 46, 65, 46 and 42 min respectively, under the HPLC conditions described in Figure 1.

Structure elucidation of the secondary metabolites

LRESIMS gave a $[M + Na]^+$ ion at *m/z* 424 for compound **1** and $[M + H]^+$ ions at *m/z* 400 and 402, for **2** and **3** respectively. Analysis of ^1H, ^{13}C and 2D NMR spectral data (gradCOSY60, gradHSQC, grad^{15}NHSQC, gradHMBC, grad^{15}NlrHMQC, tROESY) leads to the assignment of the molecular formulas $C_{24}H_{35}NO_4$, $C_{24}H_{33}NO_4$ and $C_{24}H_{35}NO_4$ for compounds **1**, **2** and **3** respectively, which were in agreement with the molecular weights of 401, 399, 401 daltons as determined by the LRESIMS. Detailed analysis of the spectral data revealed that the structures of the isolated compounds **1**–**3** matched those of the known cytochalasans, aspochalasin D, aspochalasin B and M respectively[14–16]. The structure for each compound was confirmed by comparison of the ^{13}C and ^1H spectral data with that previously reported in the literature[14–16]. The spectra obtained during the current study are given in the supporting documents while a comparison of ^{13}C values obtained for **1**–**3** in the present study and published data are given in Table 1.

Analysis of the ^1H, ^{13}C and 2D NMR spectra (gradCOSY60, gradHSQC, grad^{15}NHSQC, gradHMBC, grad^{15}NlrHMQC, tROESY) obtained for compound **4**, which gave a $[M + H]^+$ ion at *m/z* 507 in the LRESIMS, allowed for the molecular formula of **4** to be determined as $C_{32}H_{30}O_4N_2$. The spectral data revealed that the structure of the isolated compound **4** matched that of the known phenylalanine derivative, asperphenamate (**4**)[17]. The ^1H and ^{13}C NMR spectra obtained for compound **5** revealed a marked similarity to those of **4**. An additional carbon resonance at δ 54.74 along with the downfield

shift of one of the aromatic carbon resonances to δ 157.87 suggested the presence of an aromatic OMe substituent in the structure of **5**. The presence of the OMe substituent was confirmed by the LRESIMS since **5** gave a $[M + H]^+$ ion at *m/z* 537 which is 30 mass units more than that of **4** to give a molecular formula of $C_{33}H_{32}O_5N_2$ for **5**. Comparison of the ^1H and ^{13}C NMR spectra obtained for **5** with previously reported data (Table 2) revealed that the structure of compound **5** matched that of the known phenylalanine derivative, 4-OMe-asperphenamate (**5**)[18].

Antimicrobial activity of the isolated compounds

Aspochalasin D (**1**) and B (**2**) exhibited potent antibacterial activity against Gram positive *B. subtilis*, *S. aureus* and MRSA and no activity against Gram-negative *E. coli*, *P. aeruginosa* and pathogenic fungus *C. albicans*. The MIC values obtained in the current study are given in Table 3. Aspochalasin M (**3**), asperphenamate (**4**) and 4-OMe-asperphenamate (**5**) were inactive for all the tested microorganisms up to 64 μg ml^{-1}.

Discussion

Fungi in the marine ecosystem comprise both obligatory fungi and facultative fungi[5]. According to Kohlmeyer and Kohlmeyer[19], obligatory marine fungi grow and sporulate exclusively in a marine environment while facultative marine fungi have spread to the marine environment from terrestrial or freshwater habitats, but they also can exist well in the marine environment. The fungi isolated in the current study could be either obligatory or facultative 'marine fungi', hence other than using the term marine fungi, referring to them as marine/sponge-derived fungi is more appropriate as we have not determined the origin. *A. flavipes* has been reported from both terrestrial and marine sources[20–23]. Therefore, whether *A. flavipes* isolated in this study is a facultative marine fungus, that has been accumulated through filter feeding or an obligatory marine fungus, which has originated in the internal tissues, is open to conjecture.

A. flavipes is known to produce a number of secondary metabolites[24]. However, the genomic sequence data of *Aspergillus* has revealed, there is potential for new natural products to be discovered from this source[25]. Therefore, studying the secondary metabolites production by *A. flavipes* from an untapped ecological setting may prove to be fruitful. In this study, five different culture media were utilized to facilitate the growth of fastidious fungal species who seek specific nutrients and conditions to emerge.

Table 1. Comparison of ^{13}C NMR data of aspochalasin D, B and M (**1–3**) from the present study (in DMSO-d_6) with published data (in $CDCl_3$)[14–16]

	^{13}C δ (ppm) for **1**		^{13}C δ (ppm) for **2**		^{13}C δ (ppm) for **3**	
C#	Present study	Published values	Present study	Published values	Present study	Published values
1	173.5	175.1	172.1	173.6	175.3	175.3
2-NH	–	–	–	–	–	–
3	50.03	51.2	50.7	52.0	49.52	50.8
4	48.52	49.5	46.7	47.4	49.35	52.1
5	34.45	35.0	34.1	34.8	34.59	35.2
6	139.8	140.3	137.7	138.6	139.74	140.0
7	125.39	125.7	126.4	126.4	125.10	125.6
8	42.76	43.6	40.7	41.5	42.64	43.5
9	67.93	68.2	68.8	69.2	67.04	66.9
10	48.63	48.3	48.4	48.3	48.55	48.5
11	13.14	13.5	13.4	13.8	13.11	13.5
12	19.58	19.9	19.7	20.2	19.52	19.8
13	124.36	124.1	124.4	124.5	125.10	123.8
14	136.11	137.3	135.7	136.0	134.5	137.1
15	39.4	39.5	40.4	39.8	35.87	36.8
16	29.1	29.2	31.5	32.3	31.77	26.4
17	78.61	79.2	73.5	74.5	213.15	216.0
18	74.5	75.6	204.7	204.9	77.08	77.4
19	144.07	142.1	141.0	141.5	26.22	34.5
20	128.32	129.4	127.5	–	33.27	37.5
21	197.24	197.6	196.5	195.3	210.44	214.5
22	23.94	24.9	23.9	25.0	23.94	25.0
23	23.5	23.5	23.2	23.8	23.47	23.5
24	21.5	21.4	21.2	–	21.59	21.5
25	15.22	15.5	15.0	15.4	14.92	16.2

Table 2. Comparison of ^{13}C NMR data of asperphenamate (**4**) and 4-OMe-asperphenamate (**5**) from the present study (in DMSO-d_6) with published data (**4** in $CDCl_3$ and **5** in DMSO-d_6)[17,18]

	^{13}C δ (ppm) for **4**			^{13}C δ (ppm) for **5**	
C#	Present study	Published values	C#	Present study	Published values
1	138.31	137.74	1	138.3	137.3
2, 6	128.98	129.16	2, 6	129.6	130.3
3, 5	128.19	128.61	3, 5	113.6	114.3
4	126.45	126.77	4	157.87	158.9
7	36.25	37.54	7	36.23	36.7
8	49.86	54.47	8	49.84	50.4
9	65.54	65.42	9	65.44	65.4
10	–	–	10	54.74	54.6
N–H^1	–	–	N-H^1	–	–
1′	137.82	135.14	1′	134.4	134.2
2′, 6′	129.05	129.30	2′, 6′	129.04	129.4
3′, 5′	128.19	128.69	3′, 5′	128.27	128.8
4′	126.18	126.17	4′	126.17	127.2
7′	35.92	37.26	7′	35.13	37.4
8′	54.43	50.28	8′	54.9	55.2
9′	171.53	171.91	9′	171.6	172.1
N–H^2	–	–	N–H^2	–	–
1″	134.45	134.20	1″	138.3	137.2
2″, 6″	127.36	127.11	2″, 6″	127.2	127.1
3″, 5″	128.27	128.72	3″, 5″	128.2	128.5
4″	131.51	132.02	4″	130.0	131.5
7″	166.65	167.42	7″	166.18	166.7
1‴	133.63	133.32	1‴	133.63	133.4
2‴, 6‴	127.17	127.04	2‴, 6‴	127.37	127.2
3‴, 5‴	128.24	128.71	3‴, 5‴	128.2	128.5
4‴	131.15	131.39	4‴	131.5	132.1
7‴	166.19	167.21	7‴	166.67	167.5

Table 3. MIC values obtained for **1**–**5** and the positive controls

	MIC values (μg ml^{-1})					
	B. subtilis	*S. aureus*	MRSA	*E. coli*	*P. aeruginosa*	*C. albicans*
1	**32**	**32**	**32**	>64	>64	>64
2	**16**	**32**	**32**	>64	>64	>64
3	>64	>64	>64	>64	>64	>64
4	>64	>64	>64	>64	>64	>64
5	>64	>64	>64	>64	>64	>64
Polymixin B	8	–	–	4	4	–
Rifamycin	–	0.015	0.015	–	–	–
Amphotericin	–	–	–	–	–	0.062

The three fungal species isolated in this study emerged from either ME or MEA medium indicating that they prefer a medium rich with malt for their growth.

Previous reports confirm the antibacterial activity of compounds **1** and **2** (refs 15, 26). Compound **1** is reported to be active against *Arthrobacter globiformis*, *A. aurescens*, *A. oxydans*, *A. pascens*, *B. subtilis*, *Brevibacillus brevis*, *Rhodococcus erythropolis* and *S. aureus* at 1 mg ml^{-1} (ref. 26). A group of researchers have raised the question of whether the hydroxyl functionalities at C-17 to C-20 are required for antibacterial activity since aspochalasin D (**1**) exhibits activity while aspochalasin Z (absence of any oxygen functionalities between C-17 and C-20) does not[26]. The results of the current study show that compounds **1** and **2**, both of which have a hydroxyl at C-17, exhibit antibacterial activity in agreement with this argument. In another study, **1** and **2** are reported to inhibit the growth of four cancer cell lines and the authors imply that the α,β-unsaturated ketone is an essential part of the pharmacophore[14]. Compound **3** which showed no antibacterial activity lacks both the α,β-unsaturated ketone and the C-17 hydroxyl; instead, there is a hydroxyl at C-18 and a ketone at C-17. Based on the results obtained for the three aspochalasins **1**–**3** in the current study and the earlier arguments, we can suggest that the α,β-unsaturated ketone and hydroxyl group at C-17 may well be essential for the antibacterial activity. Other than the above, **2** has shown 40% inhibition of glucose transport in *A. sialophilus* while **1** has shown no inhibition[27]. Although **3** was inactive against tested bacteria and fungi, it has shown modest activity against the cancer cell line HL-60 with an IC_{50} value of 20 μM (ref. 14).

In the current study, asperphenamate **(4)** and 4-OMe-asperphenamate **(5)** were inactive against the microorganisms tested at up to 64 μg ml^{-1}. In literature some studies have shown **4** and **5** to be antibacterial while some have reported contradictory results[18,28]. In a study undertaken in China, **4** and **5** are reported to show selective activities against *S. epidermidis*, *S. aureus*, *E. coli* and *B. cereus* with MIC values of 10 μM for *S. epidermidis* and >20 μM for the other bacteria and inactive against *B. subtilis*, *Micrococcus luteus* and two marine pathogenic bacteria *Vibrio parahaemolyticus* and *Listonella anguillarum*[18]. The same study reports the absence of antifouling activity against the settlement of the larvae of the barnacle, *Balanthus amphitrite*[18]. In contrast, compound **4** isolated from *Z. digitalis* extracts showed no activity against the microorganisms *E. coli*, *S. aureus* and *Cladosporium sphaerospermum*[28]. Similarly, **4** derived synthetically failed to show any antibacterial activities against the tested microorganisms[28]. However, when **4** was isolated during a phytochemical study of *Erythrina droogmansiana*, radical scavenging potential with an IC_{50} value of 3.31 mg ml^{-1} and moderate reducing power ability was observed[29]. In another study, **4** exhibited weak *in vitro* anti proliferative activities against T47D, MDA-MB231 and HL60 cell lines[30].

Conclusion

By isolating the appealing compounds, aspochalasin B, D and M, asperphenamate and 4-OMe-asperphenamate from the fungus *A. flavipes*, the current research highlights the potential of isolating bioactive secondary metabolites from Sri Lankan sponge-derived fungi and that the marine habitat of Sri Lanka warrants further exploration for bioactive natural products.

Conflict of interest: The authors declare that they have no conflict of interest.

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