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Research Article

ANTIMICROBIAL METABOLITES FROM A MARINE-DERIVED FUNGUS

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ABSTRACT

A marine fungus was separated from the surface of a marine red algae. Ethyl acetate extract of the cultivated marine fungus inhibited the growth of the pathogenic bacteria *E.coli* and *S.aureus*, and the phytopathogenic fungus *C. cucurbitarum*. Separation of the EtOAc extract gave four compounds, which were identified as new anthraquinone derivatives **3** and **4**, and the known chrysophanol (**1**) and rubelin A (**2**) by the extensive analysis of NMR data. Compounds **1-4** inhibited the growth of the gram negative bacteria *E. coli* and gram positive bacteria *S. aureus*. Compound **3** also inhibited the fungus *C. cucurbitarum*.

Key words: marine-derived fungus, structure elucidation, anthraquinone, antimicrobial activity

INTRODUCTION

Marine microorganisms such as bacteria and fungi inhabit every environment of the sea and are rich sources of pharmacologically active compounds. The search for components with antimicrobial activity has gained increasing importance in recent times, due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms. There has also been a rising interest in the research for natural products from marine-microorganisms for the discovery of new antimicrobial agents in the last three decades (Faulkner 2002; Blunt *et al.* 2011). Fungi isolated from various organisms in the marine environment, e.g., from mangroves (Isaka *et al.* 2002), sponge (Bringmann *et al.* 2003) and particularly from algae (Abdel-Latef *et al.* 2003), have been examined for their secondary metabolites. Anthraquinone derivatives have been isolated from fungus genus *Ramularia* (Miethbauer *et al.* 2006) and these compounds showed cytotoxic and antibacterial activities (Zhou *et al.* 2006). As part of our studies on secondary metabolites from marine organisms, we have investigated the chemical constituents of an unidentified marine fungus (strain F-F-3C), isolated from the surface of a marine red algae collected at the coast of Tarama Island, Okinawa. We describe the isolation, structure elucidation and bioactivities of the two new anthraquinones (**3** and **4)** together with two known chrysophanol **(1)** (Anderson, 1985; Zhou *et al.* 2006; Miethbauer *et al.* 2008; Rani *et al.* 2010) and rubellin A (**2**) (Arnone *et al.* 1986; Miethbauer *et al.* 2008).

MATERIAL AND METHODS

General experimental procedures

The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a JEOL α-500 spectrometer. Chemical shifts were referenced to residual solvent signals ($CDCl_3$; δ_H 7.26, δ_C 77.0). Homonuclear ^{1}H connectivities were determined by the COSY experiment. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined by the HMQC experiment. Two and three-bond ^{1}H-^{13}C connectivities were determined by the gradient-HMBC. The ^{1}H connectivities through-space were elucidated by the NOE experiment. High performance liquid chromatography (HPLC) was performed on a HITACHI L-6000 pump equipped with a water RI detector (R401), using Nacalai tesque COSMOSIL packed column (5C18, 10 x 250mm and 55L, 10 x 250 mm), Merck Hibar pre-packed column (RT 250-10 RP-18, 7μm). Merck silica gel 60 (particle size 0.063-0.200mm, 70-230 mesh,

ASTM) was used for open column chromatography. Merck TLC plates (silica gel 60 F_{254} 20 x 20cm) were used for analytical and preparative TLC. Compounds were detected under UV light (254nm) and/or exposed to anisaldehyde solution [(*p*-anisaldehide (5mL), methanol (200mL), sulfuric acid (6mL), and acetic acid (2mL)], followed by heating. All solvents used were reagent grade.

Collection and cultivation

The fungus strain F-F-3C was separated from the surface of a marine red alga collected at the coast of Tarama Island, Okinawa, Japan in August 2008, and grown in fungi medium agar containing 50% sea water, 3% agar powder, 1.48% malt, 1.4% glucose, and 0.08% peptone for 17 days.

Extraction and Isolation

The sample (90 petri dishes) was extracted with acetone three times at room temperature and filtered. The extract was concentrated under reduced pressure. The resulting residue was partitioned between ethyl acetate and water to give 116mg of ethyl acetate extract. The organic layer (116mg) showed strong inhibition against the pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*, and the phytopathogenic fungi *Choanephora cucurbitarum* at 100µg/disk. Then the organic layer was subjected to open column chromatography on silica gel using mixtures of hexanes-EtOAc-MeOH as solvent systems to afford 12 fractions. Fraction 1 (6.5mg) was separated by HPLC on Si-60 with EtOAc : hexanes (1:2) solvent system to give compound **1** (1mg). Fractions 7 gave compound **3** (7.5mg). Compound **2** (6.4mg) were isolated from fraction 10 (14.2mg) by HPLC using EtOAc: hexanes (2:1), and fraction 11 (15.8mg) was separated by HPLC using EtOAc: hexanes (2:1) to yield compound **4** (6. mg).

Microbial test cultures and growth conditions

The gram negative bacterium *E. coli* and gram positive bacterium *S. aureus* were used for antibacterial tests, and *A. niger*, *Cladosporium* sp. and *C. cucurbitarum* were used for antifungal tests. Bacterial strains were maintained on bacteria medium agar (meat extract 0.05%, peptone 0.1%, NaCl 0.05%, and agar 3%) in petri dishes at 4°C, while fungi were maintained on fungi medium agar (malt extract 1.48%, glucose 1.4%, peptone 0.08% and agar 3%). Antifungal and antibacterial in-vitro assays were conducted by using the disk diffusion method. All the procedures were done according to clinical laboratory standard and quality control. The fresh cultures were obtained by growing the test strains overnight at 37°C for bacteria, while fungi were grown at 28°C for 48h.

Antimicrobial activity assay

The crude extracts and pure compounds were tested for antimicrobial activity against bacteria (*E. coli* and *S. aureus*) and fungi (*A. niger*, *C. cucurbitarum* and *Cladosporium* sp.). Prior to testing, single colonies of the microorganisms used in the bioassay, were sub-cultured in 5mL of bacteria and fungus liquid medium and incubated for 24 hours. Aliquots of the test solution were applied to sterile paper disks (8 mm diameter) using a final disk loading concentration of 25, 50, and 100µg/disk for the crude extracts, and 25 and 50µg/disk for the pure compounds. The plates were incubated at 37°C for 24h and antimicrobial activities were determined by measuring the diameter of the inhibitory zones in millimeter.

RESULTS AND DISCUSSION

Structural determination

Marine fungus strain F-F-3C was cultured in fungi agar medium for 17 days and the metabolites were extracted with acetone and then partitioned between water and ethyl acetate. The ethyl acetate extract showed strong inhibition the bacteria *E. coli* and *S. aureus* at 50µg/disk. This extract was subjected to separation by column chromatography. Bioassay guided fractionation of the active extract led to the isolation of two new anthraquinones (**3** and **4)** together with the known chrysophanol **(1)** and rubellin A (**2**) (Arnone *et al.* 1986; Zhou *et al.* 2006; Anderson, 1985; Miethbauer *et al.* 2008; Rani *et al.* 2010).

Compound **1** was isolated as a yellow oil (1 mg). ^{1}H and ^{13}C NMR correlations were demonstrated by the HMQC spectrum. ^{13}C NMR spectrum of compound **1** showed 15 carbon resonances. ^{1}H and ^{13}C-NMR and 2D NMR data of compound **1** indicated the

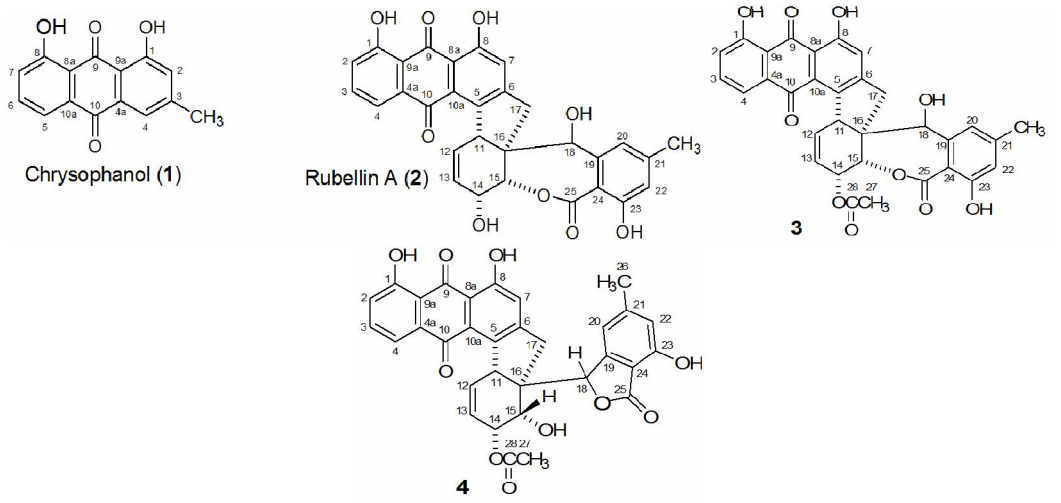

Figure 1. Compounds **1**-**4** isolated from a marine derived fungus (strain F-F-3C).

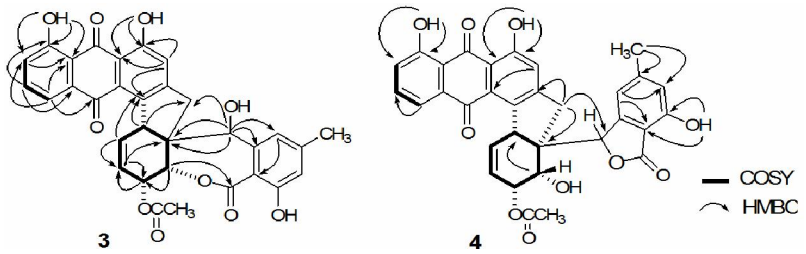

Figure 2. Key HMBC and COSY correlations of compounds **3** and **4**.

presence of twelve aromatic carbons, five aromatic protons, and two phenolic protons [δ_H7.09 (d, *J* = 1.2 Hz), δ_C 124.5 (CH); δ_H 7.64 (d, *J* = 1.2 Hz), δ_C 121.3 (CH); δ_H 7.80 (dd, *J* = 1.2, 8.0 Hz), δ_C 119.9 (CH); δ_H 7.67 (t, *J* = 8.0 Hz), δ_C 136.9 (CH); δ_H 7.27 (dd, *J* = 1.2, 8.0 Hz), δ_C 124.4 (CH); δ_C 114.0 (C); δ_C 116.0 (C); δ_C 126.2 (C); δ_C 133.0 (C); δ_C 150.0 (C); δ_C 162.4 (C); δ_C 162.7 (C); δ_{ArOH}12.02 (s); δ_{ArOH} 12.12 (s)], one methyl [δ 2.45 (s), δ_C 22.5] and two carbonyl carbons [δ_C 181.5; δ_C 191.7]. Compound **1** was confirmed to be chrysophanol by performing comprehensive NMR spectral analysis and by comparing the NMR data with those in the literature (Miethbauer *et al.* 2008; Rani *et al.* 2010).

Compound **2** was isolated as a yellow oil and the molecular formula $C_{30}H_{22}O_9$ was estimated from NMR spectral data (Tables I and II). ^{1}H and ^{13}C NMR and HMQC of **2** indicated the presence of a number of NMR signals associated with aromatic ring system [δ_H 7.28, δ_C 124.4 (CH); δ_H 7.68, δ_C 137.0 (CH); δ_H 7.79, δ_C 119.8 (CH); δ_H 7.17, δ_C 120.3 (CH); δ_H 6.90, δ_C 117.8 (CH); δ_H 6.81, δ_C 117.7 (CH); δ_C108.0 (C); δ_C 114.2 (C); δ_C 115.8 (C); δ_C 126.3 (C); δ_C 133.9 (C); δ_C 140.3 (C); δ_C 141.0 (C); δ_C 147.3 (C); δ_C 154.7 (C); δ_C 160. 8 (C); δ_C 162.2 (C); δ_C 163.5 (C); δ_{ArOH}9.38 (s); δ_{ArOH} 12.15 (s); δ_{ArOH}12.65 (s)], a disubstituted *cis* double bond [δ_H 5.75 (br d, *J* = 9.5 Hz), δ_C 129.0; δ_H 5.54 (br d, *J* = 9.5 Hz), δ_C 126.3], three oxygenated methines [δ_H 4.74 (br d, *J* = 8.5 Hz), δ_C 66.7; δ_H4.28 (d, *J* = 8.5 Hz), δ_C 86.1; δ_H 5.04 (s) , δ_C 79.5], three carbonyl carbons [δ_C 192.4, 183.2, 172.9], one methyl (δ_H 2.35, δ_C 22.2), an isolated methylene [δ_H 3.57 (d, *J* = 17.6 Hz), 3.09 (d, *J* = 17.6 Hz), δ_C 39.1], one sp^3 methine (δ_H 4.75, δ_C 47.7 and one sp^3 quarternary carbon (δ_C 54.5). Compound **2** was identified as an anthraquinone derivative rubellin A (Figure 1), which has been isolated from a fungus *Mycosphaerella rubella*, by performing comprehensive NMR spectral analysis and by comparing the NMR data with those in the literature (Arnone *et al.* 1986).

Table I. ^{1}H-NMR data for compounds **1**−**4**.

Position	δ_H (mult., *J*/Hz)[a]			
	1	**2**	**3**	**4**
1				
2	7.09 (d, 1.2)	7.28 (dd, 1.3, 8.1)	7.27 (dd, 1.0, 8.1)	7.23 (dd, 1.2, 8.3)
3		7.68 (t, 8.1)	7.67 (t, 8.1)	7.60 (t, 8.3)
4	7.64 (d, 1.2)	7.79 (dd, 1.3, 8.1)	7.77 (dd, 1.0, 8.1)	7.67 (dd, 1.2, 8.3)
4a				
5	7.80 (dd, 1.2, 8.0)			
6	7.67 (t, 8.0)			
7	7.27 (d, 1.2, 8.0)	7.17 (s)	7.17 (s)	7.13 (s)
8				
8a				
9				
9a				
10				
10a				
11	2.45 (s)	4.75 (br s)	4.75 (br s)	4.32 (br s)
12		5.75 (br d, 9.5)	5.79 (br d, 10.0)	5.78 (br d, 10.0)
13		5.54 (br d, 9.5)	5.43 (br d, 10.0)	5.36 (br d, 10.0)
14		4.74 (br d, 8.5)	5.83 (br d, 9.3)	5.45 (br d, 8.5)
15		4.28 (d, 8.5)	4.48 (d, 9.3)	4.31 (m)
16				
17		a: 3.57 (d, 17.6)	a: 3.66 (d, 17.8)	a: 3.39 (d, 19.5)
		b: 3.09 (d, 17.6)	b: 3.13 (d 17.8)	b: 3.21 (d, 19.5)
18		5.04 (br s)	5.04 (s)	5.74 (s)
19				
20		6.90 (s)	6.91 (s)	6.80 (s)
21				
22		6.81 (s)	6.82 (s)	6.69 (s)
23				
24				
25				
26		2.35 (s)	2.40 (s)	2.34 (s)
27			2.08 (s)	2.14 (s)
28				
OH-1	12.02(s)	12.65 (s)	12.63 (s)	12.55 (s)
OH-8	12.12 (s)	12.15 (s)	12.14 (s)	12.07 (s)
OH-23		9.38 (s)	9.40 (s)	7.48 (s)

[a] Data recorded at 500 MHz

Compound **3** was isolated as a yellow oil and the molecular formula $C_{32}H_{24}O_{10}$ was estimated from NMR spectral data (Tables I and II). The ^{1}H and ^{13}C NMR spectra resembled those of rubellin A (**2**). The major difference was the appearance of one acetoxyl group [δ_H 2.08 (3H, s), δ_C 21,1 (CH_3); δ_C 170.6 (C)] in **3** instead of a hydroxyl group in **2**. The acetoxyl group was positioned at C-14 on the basis of the ^{1}H and ^{13}C NMR chemical shifts of H- and C-14 [δ_H 5.83 (1H, d, *J* = 9.3 Hz), δ_C 69.0 (CH) in **3**; δ_H 4.74 (1H, d, *J* = 9.3 Hz), δ_C 66.7 (CH) in **2**]. The structure of **3** was determined by the interpretation of the 1D and 2D NMR data (COSY, HMQC and HMBC, Figure 2) and by comparison of the ^{1}H and ^{13}C NMR data with those of rubellin A (**2**) (Arnone *et al.* 1986). Thus, compound **3** was 14-acetate of rubellin A.

Table I. ^{1}C-NMR data for compounds **1**−**4**.

Position	δ_C (mult.)[a]			
	1	**2**	**3**	**4**
1	162.7 (s)	162.2 (s)	162.3 (s)	162.2 (s)
2	124.5 (d)	124.4 (d)	123.8 (d)	124.4 (d)
3	150.0 (s)	137.0 (d)	136.9 (d)	136.9 (d)
4	121.3 (d)	119.8 (d)	119.8 (d)	119.8 (d)
4a	126.2 (s)	133.9 (s)	133.8 (s)	133.6 (s)
5	119.9 (d)	141.0 (s)	140.9 (s)	139.2 (s)
6	136.9 (d)	154.7 (s)	154.5 (s)	153.9 (s)
7	124.4 (d)	120.3 (d)	120.4 (d)	120.9 (d)
8	162.4 (s)	163.5 (s)	163.5 (s)	163.5 (s)
8a	116.0 (s)	114.2 (s)	114.2 (s)	114.5 (s)
9	191.7 (s)	192.4 (s)	192.4 (s)	192.4 (s)
9a	114.0 (s)	115.8 (s)	115.8 (s)	115.8 (s)
10	181.5 (s)	183.2 (s)	183.2 (s)	183.2 (s)
10a	133.0 (s)	126.3 (s)	126.2 (s)	126.1 (s)
11	22.5 (q)	47.7 (d)	47.4 (d)	47.3 (d)
12		129.0 (d)	130.1(d)	129.3 (d)
13		126.3 (d)	124.4 (d)	124.4 (d)
14		66.7 (d)	69.0 (d)	73.9 (d)
15		86.1 (d)	82.0 (d)	71.9 (d)
16		54.5 (s)	54.5 (s)	53.5 (s)
17		39.1 (t)	39.1 (t)	36.6 (t)
18		79.5 (d)	79.3 (d)	86.6 (d)
19		140.3 (s)	140.0 (s)	146.5 (s)
20		117.8 (d)	117.8 (d)	116.4 (d)
21		147.3 (s)	147.3 (s)	148.7 (s)
22		117.7 (d)	117.7 (d)	116.0 (d)
23		160.8 (s)	160.6 (s)	156.4 (s)
24		108.0 (s)	108.1 (s)	110.3 (s)
25		172.9 (s)	172.3 (s)	167.0 (s)
26		22.2 (q)	22.2 (q)	22.5 (q)
27			21.1 (q)	21.3 (q)
28			170.6 (s)	170.6 (s)
OH-1				
OH-8				
OH-23				

[a] Data recorded at 125 MHz

Compound **4** was isolated as a yellow oil and the molecular formula $C_{32}H_{24}O_{10}$ was estimated from NMR spectral data (Tables I and II). ^{1}H and ^{13}C-NMR correlations were demonstrated by the HMQC spectrum. ^{1}H and ^{13}C NMR and 2D NMR data of compound **4** indicated the presence of one acetoxyl group [δ_H2.14 (3H, s), δ_C 21.3 (CH_3); δ_C 170.6 (C)], 1, 2, 3-trisubstituted, pentasubstituted and 1, 2, 3, 5- (or 1, 2, 4, 5-) tetrasubstituted benzene rings, and three phenolic protons [δ_C 162.2 (C), δ_{ArOH} 12.55 (s); δ_H 7.23 (1H, dd, J = 1.2, 8.3 Hz), δ_C 124.4 (CH); δ_H 7.60 (1H, t, J = 8.3 Hz), δ_C 136.9 (CH); δ_H 7.67 (1H, d, J = 1.2, 8.3 Hz), δ_C 119.8 (CH); δ_C 133.6 (C); δ_C 115.8 (C); 139.2 (C); 153.9 (C); δ_H 7.13 (1H, s), δ_C 120.9 (CH); δ_C 163.5 (CH), δ_{ArOH}12.07 (s); 114.5 (C); 126.1 (C); 146.5 (C); δ_H 6.80 (1H, s), δ_C 116.4 (CH);

Table III. Antibacterial and antifungal activities of crude extracts of an unidentified marine fungus F-F-3C.

Extracts	*E. coli*			*S. aureus*			*A. niger*			*Cladosporium* sp.			*C. cucurbitarum.*		
	25	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**
Acetone	-	+	++	-	+	++	-	-	-	-	-	-	-	-	-
EtOAc	+	+	++	+	+	++	-	-	-	-	-	-	-	-	+

Diameter of inhibition zone (mm); + (11-12.5), ++ (13-15.5), - (no activity);
Diameter of paper disk; 8mm
Concentration: (μg/disc)

Table IV. Antibacterial and antifungal activity of compounds (**1-4**) isolated from an unidentified marine fungus F-F-3C.

Compounds	*E. coli*		*S. aureus*		*A. niger*		*Cladosporium* sp.		*C. cucurbitarum*	
	25	**50**	**25**	**50**	**25**	**50**	**25**	**50**	**25**	**50**
1	+	++	+	++	-	-	-	-	-	-
2	+	++	+	++	-	-	-	-	-	-
3	+	++	+	++	-	-	-	-	-	+
4	+	++	+	++	-	-	-	-	-	-

Diameter of inhibition zone (mm); + (11-12.5), ++ (13-15.5), - (no activity)
Diameter of paper disk; 8 mm
Concentration: (μg/disk)

148.7 (C); δ_H 6.09 (1H, s), δ_C 116.0 (CH); 156.4 (C), δ_{ArOH}7.48 (s); 110.3 (C)], a disubstituted *cis* double bond [δ_H 5.78 (br d, J = 10.0 Hz), δ_C 129.3; δ_H 5.38 (br d, J = 10.0 Hz), δ_C 124.4], three oxygenated methines [δ_H5.45 (1H, br d, J = 8.5 Hz), δ_C 73.9; δ_H 5.74 (1H, s), δ_C 86.6; δ_H 4.31 (1H, m), δ_C 71.9], three carbonyl carbons [δ_C 192.4 (C); 183.2 (C), 167.0 (C)], one methyl [δ_H 2.34 (3H, s), δ_C 22.5], one sp^3 methylene [δ_H 3.39 (1H, d, J = 19.5 Hz), δ_H 3.21 (1H, d, J = 19.5 Hz), δ_C 36.6], one sp^3 methine [δ_H 4.32 (1H, br s), δ_C 47.3] and one sp^3 quarterny carbon [δ_C 53.5]. The NMR spectra of **4** were similar to those of anthraquinone derivertives rubellins A, and B (Arnone *et al.* 1986), and 14-dehydrorubellin D (Miethbauer *et al.* 2006). A hydroxyl group in rubellin C (Heiser *et al.* 2004; Miethbauer *et al.* 2008) was found to be replaced by an acetoxyl group in **4**. The acetoxyl group was positioned at C-14 on the basis of the ^{1}H and ^{13}C NMR chemical shifts of H- and C-14 [δ_H5.45 (1H, d, J = 4.3 Hz), δ_C 73.9 (CH)]. The structure of **3** was established by the interpretation of the 1D and 2D NMR data (COSY, HMQC and HMBC, Figure 2) and by comparison of the ^{1}H and ^{13}C NMR data with those of rubellins A and B, and 14-dehydrorubellin D. Thus, compound **4** was 14-acetate of rubellin C.

Biological activity

The antibacterial and antifungal assays were carried out at the concentration of 25 and 50μg/disk for pure compounds and 25, 50, and 100 μg /disk for crude extracts (Tables III and IV).

The EtOAc extract of an unidentified marine fungus (strain F-F-3C) showed activity against *E. coli* and *S. aureus* at 25 and 50μg/ disk, and showed antifungal activity against the fungi *C. cucurbitarum* at 100μg/disk (Table III). Compounds **1-4** inhibited the gram negative *E. coli* and gram positive bacteria *S. aureus* (13-15.5mm) at 50μg/disk. Compound **3** showed activity against the fungus *C. cucurbitarum* (11-12.5mm) at 50μg/disk (Table IV). Miethbauer *et al* (2008) reported that compounds **1** and **2** are potent cytotoxic and antibacterial agents.

CONCLUSIONS

Two new anthraquinone derivatives (**3** and **4**), and the known chrysophanol (**1)** and rubelin A (**2**) were isolated from a marine

derived fungus and their structures were elucidated by the extensive analysis of NMR data. These metabolites exhibited moderate antibacterial activity against the gram-negative bacterium *E. coli* and the gram-positive bacterium *S. aureus*. Compound **3** also showed activity against the pathogenic fungus *C. cucurbitarum*.

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