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A new sulfated triterpene glycoside from the sea cucumber *Colochirus quadrangularis*, and evaluation of its antifungal, antitumor and immunomodulatory activities

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ABSTRACT

Our continuing search for marine bioactive secondary metabolites led to the screening of crude extracts of sea cucumbers by the model of *Pyricularia oryzae*. A new sulfated triterpene glycoside, coloquadranoside A (**1**), together with four known triterpene glycosides, philinopside A, B, E and pentactaside B (**2**–**5**) were isolated from the sea cucumber *Colochirus quadrangularis*, and their structures were elucidated using extensive spectroscope analysis (ESI-MS, 1D and 2D NMR) and chemical methods. Coloquadranoside A possesses a 16-acetyloxy group in the holostane-type triterpene aglycone with a 7(8)–double bond, a double bond (25,26) at its side chain, and two *β*-D-xylose in the carbohydrate chain. Coloquadranoside A exhibits *in vitro* some antifungus, considerable cytotoxicity (IC_{50} of 0.46–2.03 μM) against eight human tumor cell lines, *in vivo* antitumor, and immunomodulatory activity.

1. Introduction

Triterpene glycosides (saponins) are the predominant secondary metabolites of sea cucumbers. More than 700 of them are described from various species of holothurians existing chemical structures of sulfated, non-sulfated and acetylated glycosides, characterized by the tremendous structural diversity.[1–3] A broad spectrum of biological activities are exhibited in them, such as hemolysis, cytotoxicity, antifungus, antibacterium, antivirus, antiinflammation, cholesterol-lowering, immunoregulation, antioxidation, antitumor and antidementia.[3–10] Therefore, the search for new representatives of these triterpene glycosides and studies of their biological activities appear to be relevant. The majority of the glycosides are usually the lanostane with an 18(20)-lactone (i.e. the holostane-type triterpene) and with a sugar chain of up to six monosaccharide units [principally D-glucose (Glu), D-xylose (Xyl), D-quinovose (Qui), and their methylated forms] linked to C-3 of the aglycone.[11] As a rule, the first sugar is identified as xylose, whereas the methylated monosaccharides are always the terminal sugars. The carbohydrate chains are sulfated, at least at C-4 of the first xylose residue. Moreover, the glycosides sulfated at C-6 hydroxy groups of glucose and 3-*O*-methylglucose, were proved to be a universality, although other positions of sulfates in different sugars rarely occur.[12–15]

As part of our ongoing search for new bioactive compounds from holothurians,[16–24] we have already described the isolation and the structure elucidation of a number of glycosides thoroughly from the sea cucumber *Colochirus quadrangularis* [16–20] (known formerly as its synonym, *Pentacta quadrangularis*) collected from the South China Sea, near Guangdong Province, China.[25,26] These glycosides usually form an extremely complicated mixture in the organism producer, and the reinvestigation aims to purify, characterize and evaluate these congeners from *C. quadrangularis*. We report herein the isolation and structural elucidation of a new triterpene glycoside from *C. quadrangularis*, coloquadranoside A (**1**), and its bioactivities *in vitro* and *in vivo*.

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2. Results and discussion

2.1. Chemical investigation

The EtOH extract of *C. quadrangularis* (5 kg, dry weight) was sequentially passed through column chromatography (DA-101 resin and silica gel), and the fraction containing coloquadranoside A (**1**) was obtained. The new compound **1** and other known compounds **2**–**5** (philinopside A, B, E and pentactaside B) were further isolated and purified by reversed-phase HPLC on Zobax SB C-18 (Fig. 1).

Coloquadranoside A (**1**), a white amorphous powder, was positive in the Liebermann-Burchard and Molish tests. Its molecular formula was determined as $C_{55}H_{85}NaO_{25}S$ from pseudomolecular ion peak at *m/z* 1223 $[M+Na]^+$ (positive-ion mode ESI-MS) and *m/z* 1177 $[M-Na]^-$ (negative-ion mode ESI-MS). The IR spectrum showed the presence of hydroxyl (3419 cm^{-1}), carbonyl (1747 cm^{-1}), and olefinic (1647 cm^{-1}) groups.

The ^1H- and ^{13}C NMR spectra of compound **1** suggested the presence of a triterpene aglycone with two olefinic bonds, one acetoxyl and one lactone carbonyl group, one hydroxyl group bonded to an oligosaccharide chain composed of four sugar units. Comparing the spectral data of **1** (Table 1) with those of the known compounds indicated that its aglycone part was identical to that of philinopside B (**3**).[16] Two C-atoms of **1** at δ_C 145.6 (C-25) and δ_C 110.6 (C-26), along with two olefinic signals at δ_C 38.2 and 22.0 (C-24 and C-27), confirmed the presence of a characteristic C(25) = C(26) bond. The ^1H- and ^{13}C NMR spectra of **1** showed resonances for a 7(8)-double bond (δ_C 145.5, C-8 and 120.1, C-7; δ_H 5.74 *br.s*, H-7) and 16β-acetyloxy group (δ_C 170.2 and 21.3, CH_3; δ_H 1.98, s) closely related to those of frondoside D isolated from *Cucumaria frondosa* and philinopside A (**2**).[16,27] Location of the AcO group at C-16 was deduced from chemical shift of the H-16 signal at δ_H 6.00 (ddd, J = 7.8, 8.4, 9.0), which showed coupling to signals at δ_H 2.69 (d, J = 9.3, H-17), 2.66 (dd, J = 8.1, 12.0, H_α-15), and 1.84 (m, H_β-15) in the ^1H–^1H COSY plot, and correlation with the carbonyl carbon signal at δ 170.2 in the HMBC spectrum. The 16β configuration of the AcO group was confirmed by NOESY and the coupling constants for H-16 with H_α-17 (9.3 Hz) and H_α-15 (8.1 Hz), which was also close to the data of pentactaside B (**5**),[19] and different with those of philinopside E (**4**) without the 16β-acetyloxy.[17] Analysis of ^1H–^1H COSY, HMQC and NOESY data allowed the assignment of all ^1H- and ^{13}C NMR resonances and established the relative configuration of all chiral centers of the aglycone. Thus, the aglycone of **1** was determined to be 16β-acetoxy-holosta-7,25-diene-3β-ol.

The sugar portion of **1** displayed ^1H- and ^{13}C NMR resonances, suggesting the presence of four monosaccharide units (four anomeric C-atoms at δ_C 105.2, 105.4, 105.1, 105.6, and four anomeric protons at δ_H 4.81, 5.12, 4.96, 5.46). The large vicinal coupling constants ($^3J_{H\text{-}1/H\text{-}2}$ = 7.1–7.9 Hz) of each anomeric proton indicated a *trans*-diaxial orientation with respect of their coupling partners (β-configuration).[4] Compound **1** was treated with aqueous 2 mol/L trifluoroacetic acid to give D-Xyl, D-Qui and D-3-*O*-methylglucose (Glu3Me) in the ratio 2: 1: 1. The monosaccharides were identified by GC–MS in the form of corresponding standard aldononitrile peracetates. Fragments corresponding to the loss of sugars and SO_3Na^+ from the $[M+Na]^+$ peak were also observed at 1121 $[M-SO_3Na+Na+H]^+$, 945 $[M-SO_3Na+Na+H$ - Glu3Me$]^+$, 791 $[M-SO_3Na+2H$ - Glu3Me - Xyl$]^+$, and 667 $[M-SO_3Na+2H$ - Glu3Me - Xyl - Qui$]^+$ showing that its Glu3Me must be terminal (Fig. 2). The common D-configuration for the four carbohydrate units were assumed also according to those most often encountered among triterpene glycosides from sea cucumbers.[4] The ^1H–^1H COSY experiment allowed the sequential assignment of most of the resonances for each sugar ring, starting from the easily distinguished signals due to anomeric protons. Complete assignment was achieved by combination of ^1H–^1H COSY and TOCSY results. The HMQC experiment correlated all proton resonances with those of their corresponding carbons. Data from the above experiments indicated (Table 2) four sugar residues existing in their pyranose forms. The location of the interglycosidic linkages was deduced from the chemical shifts of Xyl^1 C-2 (δ_C 83.6), Qui^2 C-4 (δ_C 86.2) and Xyl^3 C-3 (δ_C 87.3), which were downfield relative to shifts expected for the corresponding methyl glycopyranosides. These evidences suggested that **1** possesses the same tetrasaccharide chain as philinopside A (**2**) and philinopside E (**4**).[16,17] Finally, direct support of the sequence of the sugars and binding sites came from the results of HMBC experiment: a cross peak between C-3 of the aglycone and H-1 of Xyl^1 indicated that Xyl^1 was connected to C-3 of the aglycone; the linkage of Qui^2 at C-2 of Xyl^1 was indicated by the cross peak Qui^2 H-1/Xyl^1 C-2. Similarly, the linkages of the terminal $Glu3Me^4$ at the C-3 of Xyl^3 in turn linked to C-4 of Qui^2 were indicated by cross peaks $Glu3Me^4$ H-1/Xyl^3 C-3, Xyl^3 H-1/Qui^2 C-4. This conclusion was confirmed by the correlations between H-3 of aglycone and Xyl^1 H-1, between Qui^2 H-1 and Xyl^1 H-2, between Xyl^3 H-1 and Qui^2 H-4, and between $Glu3Me^4$ H-1 and Xyl^3 H-3, in the NOESY spectrum (Fig. 3). Fragment ion peak at *m/z* 1121 $[M-SO_3Na+Na+H]^+$ in ESI-MS indicated the presence of a sulfate groups in **1** which was confirmed by the IR spectrum with absorption bands at 1233 cm^{-1}. The site of the sulfo group linked to the sugar unit in **1** was also demonstrated by its solvolysis with dioxane/pyridine to desulfocoloquadranoside A (**1a**). The sugar moiety of **1a** was identical to that of desulfated intercedenside A from *Mensamria interceden* and desulfophilinopside B from *C. quadrangularis*.[17,28] When the ^{13}C NMR signals of the sugar moiety of **1** were compared with those of **1a**, an

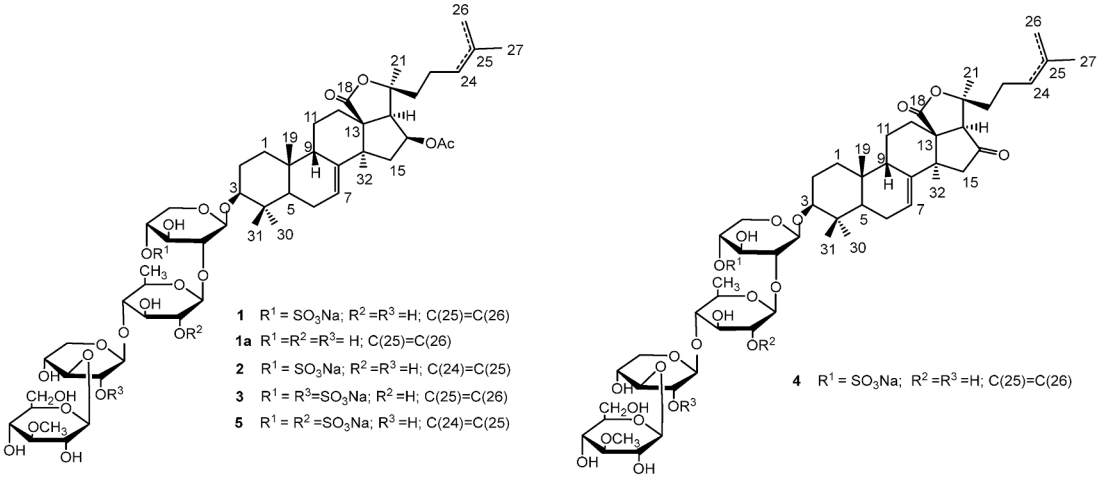

Fig. 1. Structure of compounds, extracted from *C. quadrangularis*. (The new compound in this paper: **1**, coloquadranoside A; the related structurally known compounds [16,17,19]: **2**, philinopside A; **3**, philinopside B; **4**, philinopside E; **5**, pentactaside B).

Table 1
^{1}H- and ^{13}C NMR data and select HMBC correlations for the aglycon moiety of coloquadranoside A (**1**) (δ in ppm, J in Hz).[a]

Position	δ_C[a]	δ_H (J in Hz)[b]	^{1}H-^{1}HNOESY	HMBC (H → C)
1	36.2, CH_2	1.42 (m)		
2	27.4, CH_2	1.87–2.05 (m)	H-19	
3	89.3, CH	3.33 (dd, J = 4.2, 11.4)	H-5, H-31, H_α-1	C-1 (Xyl^1)
4	39.5, C			
5	48.1, CH	0.99 (t, J = 7.8)	H_α-3, H-31, H-7	C-4, C-30, C-31
6	23.4, CH_2	2.00 (m)		
7	120.1, CH	5.74 (br. s)	H-17, H-31, H-32, H-5	
8	145.5, C			
9	47.2, CH	3.56 (bd, J = 14.2)		
10	36.0, C			
11	22.5, CH_2	1.51–1.76 (m)		
12	31.6, CH_2	2.06–2.25 (m)		
13	59.3, C			
14	47.5, C			
15	43.7, CH_2	1.84 (m, H_β); 2.66 dd (8.1, 12.0, H_α)		
16	75.1, CH	6.00 (ddd, J = 7.8, 8.4, 9.0)	H_α-17, H-32	C-17, MeCO
17	54.7, CH	2.69 (d, J = 9.3)	H_α-16, H-7, H-32, H-21	C-13, C-21
18	179.6, C			
19	24.1, CH_3	1.31 (s)	H_β-2, H-30,	
20	85.1, C			
21	28.3, CH_3	1.66 (s)	H-17	
22	38.4, CH_2	1.78–2.33 (m)		
23	22.8, CH_2	1.95–2.04 (m)		
24	38.2, CH_2	1.97 (t, J = 7.8)		C-26, C-27
25	145.6, C			
26	110.6, CH_2	4.77 (t)		
27	22.0, CH_3	1.67 (s)		
30	17.2, CH_3	1.13 (s)	H-19	C-3, C-5, C-31
31	28.7, CH_3	1.23 (s)	H-3, H-5, H-7	C-3, C-5, C-30
32	32.5, CH_3	1.04 (s)	H-16, H-17, H-7	C-8, C-13, C-14
MeCO	170.2,			
MeCO	21.3,	1.98 (s)		

[a] Measured at 150 MHz in C_5D_5N/D_2O 4:1.
[b] Measured at 600 MHz in C_5D_5N/D_2O 4:1.

esterification shift (from 70.8 to 75.4) (Table 3) was observed at the signals of C-4 (Xyl^1). Data from NOESY and HMBC experiments were combined to check the sequence of sugars, which was also indicated by ESI-MS, and to determine the attachment points of interglycosidic. The sequence of the sugar residues of **1**, determined from HMBC cross-peaks at H-1′/C-3, H-1″/C-2′, H-1‴/C-4″, and H-1⁗/C-3‴, should be as Glu3Me-(1 → 3)-Xyl-(1 → 4)-Qui-(1 → 2)-Xyl-(1 → 3)-aglycone.

The structure of compound **1** was finally elucidated on the basis of extensive spectroscopic analysis and chemical evidence as 16β-acetoxy-3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-β-D-quinovopyranosyl-(1 → 2)-4-*O*-sodiumsulfate-β-D-xylopyranosyl]-holosta-7,25-diene-3β-ol.

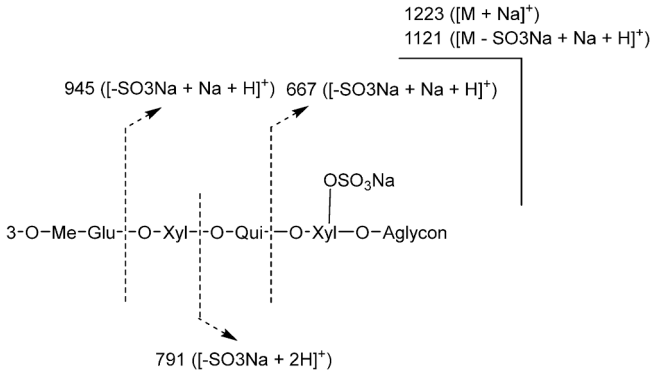

Fig. 2. Sketch map of ESI-MS fragmentation of coloquadranoside A (**1**) with positive-ion mode.

Table 2
^{1}H- and ^{13}C NMR data and key HMBC correlations for the sugar moiety of coloquadranoside A (**1**) (δ in ppm, J in Hz).

Position	δ_C[a]	δ_H[b]	HMBC (H → C)
4-$NaSO_3$-Xyl^1 (1 → C-3)			
1	105.2, CH	4.81 (d, J = 7.2)	C-3 (Aglycone)
2	83.6, CH	4.05–4.13 (m)	
3	76.4, CH	4.01–4.12 (m)	
4	75.4, CH	5.17–5.24 (m)	
5	64.3, CH_2	3.79–3.86 (m)	
		4.70–4.78 (m)	
Qui^2 (1 → 2)			
1	105.4, CH	5.12 (d, J = 7.6)	C-2 (Xyl^1)
2	75.6, CH	3.99–4.10 (m)	
3	75.8, CH	4.28–4.36 (m)	
4	86.2, CH	3.66–3.70 (m)	
5	71.9, CH	4.03–4.11 (m)	
6	18.0, CH_3	1.83 (d, J = 6.2)	
Xyl^3 (1 → 4)			
1	105.1, CH	4.96 (d, J = 7.8)	C-4 (Qui^2)
2	74.0, CH	3.78–3.86 (m)	
3	87.3, CH	4.23–4.28 (m)	
4	69.1, CH	4.15–4.23 (m)	
5	66.5, CH_2	4.37 (m, H_α)	
		3.72–3.77 (m, H_β)	
Glu3Me^4 (1 → 3)			
1	105.6, CH	5.46 (d, J = 7.9)	C-3 (Xyl^3)
2	75.1, CH	4.27–4.35 (m)	
3	88.2, CH	3.73–3.83 (m)	
4	70.5, CH	4.10–4.19 (m)	
5	78.4, CH	4.05–4.13 (m)	
6	62.0, CH	4.26–4.36 (m); 4.55 (m)	
OMe	54.7	3.86 (s)	C-4 (Xyl^3)

[a] Measured at 150 MHz in C_5D_5N/D_2O 4:1.
[b] Measured at 600 MHz in C_5D_5N/D_2O 4:1.

2.2. Antifungal activity and cytotoxicity

Some triterpene glycosides hitherto isolated from sea cucumbers showed antifungal activity.[29] Compounds **1**–**5**, extracted from *C. quadrangularis*, exhibited selective antifungal activities against four strains, while **1**, **3** and **4** possessing C(25) = C(26) showed more considerable growth inhibitory activities among them, with MIC_{80} of 4–16 μg·mL^{-1} (Table 4). Cytotoxicity of compounds **1**–**5** against eight human tumor cell lines (A-549, MKN-28, MCF-7, HCT-116, BEL-7402, HO-8910, HL-60, SPC-A4) *in vitro* were examined using the sulforhodamine (SRB) assay with HCP as a positive control.[30] It was interesting that **1** and **3** with the both presence of C(25) = C(26) and AcO exhibited more considerable cytotoxicity against some tumor cell lines (Table 5) after exposures. As previous studies reported, the membranolytic activity plays a substantive role for cytotoxicity and antifungal activity of the lanostane glycosides with an 18(20) lactone and a sugar chain linked

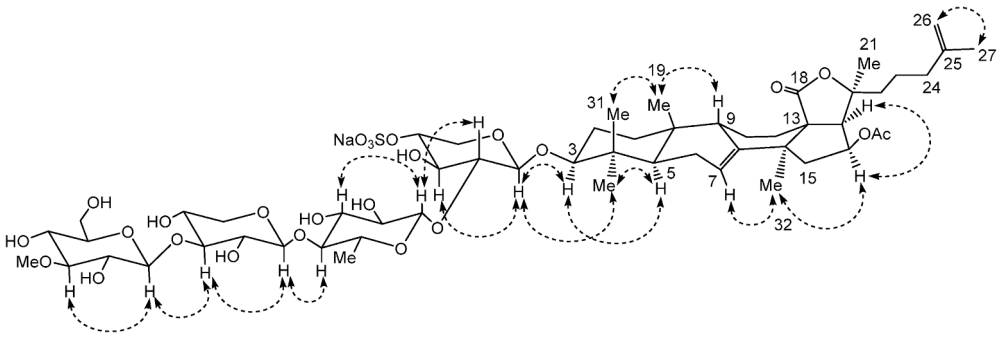

Fig. 3. Key NOESY correlations for coloquadranoside A (**1**), while its absolute configuration remains to be determined.

Table 3
^{13}C NMR data for the aglycon and sugar moiety of desulfocoloquadranoside A (**1a**) (δ in ppm, J in Hz).

Aglycon	δ_C[a]	Sugar unit	δ_C[a]
1	36.2, CH_2	Xyl^1 (1 → C-3)	
2	27.3, CH_2	1	105.1, CH
3	89.2, CH	2	84.5, CH
4	39.6, C	3	78.6, CH
5	48.0, CH	4	70.8, CH
6	23.4, CH_2	5	66.8, CH_2
7	120.5, CH		
8	145.6, C	Qui^2 (1 → 2)	
9	47.0, CH	1	105.5, CH
10	35.8, C	2	76.6, CH
11	22.7, CH_2	3	75.6, CH
12	31.5, CH_2	4	86.1, CH
13	59.2, C	5	71.7, CH
14	47.4, C	6	17.6, CH_3
15	43.7, CH_2	Xyl^3 (1 → 4)	
16	74.9, CH	1	105.5, CH
17	54.6, CH	2	73.7, CH
18	179.5, C	3	87.3, CH
19	24.0, CH_3	4	69.1, CH
20	85.2, C	5	66.6, CH_2
21	28.1, CH_3		
22	38.7, CH_2	Glu3Me^4 (1 → 3)	
23	23.8, CH_2	1	105.4, CH
24	38.3, CH_2	2	75.1, CH
25	145.5, C	3	88.0, CH
26	110.3, CH_2	4	70.8, CH
27	18.1, CH_3	5	78.5, CH
30	17.3, CH_3	6	62.2, CH
31	28.5, CH_3		
32	32.1, CH_3	OMe	60.7
MeCO	169.9		
MeCO	21.1		

[a] Measured at 150 MHz in C_5D_5N/D_2O 4:1.

Table 4
Antifungal activities of compounds (MIC_{80}, μg·mL^{-1}).

Compounds[a]	*C. albicans* ATTCC76615	*C. neoformans* ATTCC32609	*C. tropicalis*	*C. parapsilosis*
1	4	16	8	4
2	20	25	30	32
3	4	16	8	4
4	4	16	8	4
5	25	25	25	25
ICZ	<0.125	2	1	<0.125
TRB	32	<0.125	<0.125	<0.125
FCZ	0.25	2	1	0.5

[a] Itraconazole (ICZ), Terbinafine (TBNF) and Fluconazole (FCZ) were used as pos. controls.

to C-3 of the aglycone,[1,2] and the component of the carbohydrate chain and the double bond position in the aglycone moiety, as well as the side chains, are assumed to be important for cytotoxic activity among these glycosides against human tumor cells.[19–23,28] Our analysis suggested that the Δ^{25} terminal double bond and 16β-acetyloxy group of coloquadranoside A (**1**) contribute to its cytotoxicity and antifungal activity. Based on these initially promising results, coloquadranoside A deserves further study as a potential antifungal and antitumor compound, although more extensive studies are needed before a clear structure-activity relationship can be reached.

2.3. Antitumor activity in vitro

When tested for bioactivity by inducing morphological deformation of mycelia (Table 6), coloquadranoside A showed deformation against *P. oryzae* with MMDC ≤ 16 μg/mL.[31,32] It indicated that the compound has the potential antitumor probably, consistent with its observation in cytotoxicity assay. Then, HCT-116 was selected to characterize its antitumor activity *in vitro*. Transwell migration assay demonstrated that the migration capacity of the 24 h treated cells was significantly diminished after administration (Fig. 4). Hoechst results revealed that when exposed to coloquadranoside A, the cells underwent the typical morphological changes that are associated with apoptosis, as indicated by dense granular fluorescence (Fig. 4). The cells in vehicle group rarely exhibited the apoptotic nuclei, while the apoptotic nuclei cells were easily seen in the coloquadranoside A groups (Fig. 4). Quantitatively, the compound significantly increased the number of apoptotic nuclei in HCT-116 cells. Besides, the apoptosis induced by coloquadranoside A was further confirmed by Annexin V and propidium iodide double-staining assay. It showed that the compound significantly decreased the percentages of live cells (Q_4) with increased percentages of the early and late apoptotic cells (Q_3 and Q_2) and necrotic cells (Q_1) in HCT-116 cells (Fig. 4). And thereby it was evident that the compound induces apoptosis in cancer cells. The overall results demonstrated that coloquadranoside A has the ability to suppress the migration of cancer cells, meanwhile accelerating their apoptosis, revealing its potential antitumor activity *in vitro*.

We confirmed the antitumor activity of coloquadranoside A preliminarily, while the extracorporeal angiogenesis model results also demonstrated it obviously inhibited the proliferation, migration and tube formation of HMEC-1 s (Table 7). Angiogenesis is closely related to the occurrence and development of tumors, as the guarantee of tumor growth and metastasis.[33] The results indicated the antitumor role of coloquadranoside A through directly inhibition of tumor cell growth and meanwhile probably suppressing tumor angiogenesis and formation of microvascular neovascularization. Furthermore, our observation demonstrated it suppressed the autophosphorylation of VEGFR-2 (IC_{50} of 2.20 μM) and PDGFR-β (IC_{50} of 4.80 μM), two types of tyrosine kinase receptors, both of which are closely related to angiogenesis.[34–36] In sum, coloquadranoside A possesses the potential in suppressive activity of tyrosine kinase receptors and probably inhibits tumor growth and

Table 5
Cytotoxicity of compounds against human tumor cells *in vitro* (IC_{50}, μM).

Compounds	A-549	MKN-28	MCF-7	HCT-116	BEL-7402	HO-8910	HL-60	SPC-A4
1	1.27 ± 0.14	1.83 ± 1.01	2.03 ± 0.20	1.36 ± 0.09	1.45 ± 0.11	1.94 ± 0.35	0.60 ± 0.30	1.50 ± 0.70
2	1.70 ± 0.99	2.81 ± 0.50	2.30 ± 0.51	1.61 ± 0.42	2.40 ± 0.12	2.32 ± 0.50	1.65 ± 0.32	3.67 ± 0.72
3	0.75 ± 0.23	2.03 ± 0.32	1.79 ± 0.21	0.93 ± 0.01	1.30 ± 0.11	1.90 ± 0.31	0.65 ± 0.20	1.30 ± 0.71
4	1.47 ± 0.26	5.53 ± 0.19	3.03 ± 0.10	1.01 ± 0.21	1.75 ± 0.23	2.14 ± 0.22	0.82 ± 0.29	3.65 ± 0.21
5	1.30 ± 0.13	1.82 ± 0.09	1.56 ± 0.22	1.09 ± 0.02	1.30 ± 0.21	1.90 ± 0.41	1.95 ± 0.20	1.50 ± 0.71
HCP[a]	0.74 ± 0.04	0.48 ± 0.04	0.90 ± 0.04	0.14 ± 0.01	0.27 ± 0.03	0.70 ± 0.21	0.41 ± 0.23	0.66 ± 0.23

[a] HCP, 10-hydroxycamptothecine (pos. control).

Table 6
Effects of compounds against *P. oryzae*.

Compounds	**1**	5-FU[b]	HCP[b]
MMDC (μg/mL)[a]	≤ 16	≤ 8	≤ 5

[a] Compounds with significant activity (MMDC ≤ 125 μg/ml) are in bold; MMDC, minimum deformative concentration.
[b] 5-FU, 5-fluorouracil; HCP, 10-hydroxycamptothecine (pos. control).

aggressiveness by suppressing tumor neovascularization through tyrosine kinase autophosphorylation pathway.

2.4. Potential for suppressing tumors in vivo

Because coloquadranoside A displayed potent antitumor effects *in vitro*, the activity *in vivo* was tested in mice next. We established two homograft tumors in the wild-type mice successfully (Fig. 5), S-180 and H22 models. An ANOVA revealed that 5-FU significantly inhibited the weight gaining of tumors in the homograft mice ($P < 0.05$). The inhibition was also found on the groups of coloquadranoside A ($P < 0.01$), suggesting its same ability as 5-FU against the growth of S-180 sarcomas and H22 tumors *in vivo*. Meanwhile, TGI of coloquadranoside A with the dose of 5–50 mg/kg reached more than 35% ($P < 0.01$, Table 8), showing valid. Based on its effective inhibition on the growth of two homograft tumors, the antitumor efficacy of coloquadranoside A *in vivo* was proved. These preliminary results revealed coloquadranoside A is warranted to be further studied on antitumor.

For further explanation of the antitumor activity, the xenograft model of human hepatocarcinoma BEL7402 was established to assay the effectiveness of coloquadranoside A in the SPF grade immunodeficiency BALB/c-nu mice. As the data of the last day shown, the BEL7402 xenograft mice model was successful (Fig. 5). The increase of average tumor volume in vehicle group was exponential in the last few days, while it tended to slow due to coloquadranoside A treatment (Fig. 5). The ANOVA revealed that the last-day average RTV and average tumor weight were significantly lower after treatment ($P < 0.05$; Fig. 5) with TGI > 50% and T/C < 60% ($P < 0.05$; Table 8), which suggested their effectiveness. Taken together, the most obvious finding was that coloquadranoside A exhibits an excellent antitumor activity against three graft tumors *in vivo*, mainly through reducing the weight and volume of tumors, and the evaluating indicator like TGI and T/C confirmed our observation. It may support the hypothesis that coloquadranoside A has the potential to effectively suppress the growth, aggressiveness and angiogenesis of a series of tumors, and is expected to become an antitumor lead compound and novel medication just as two positive controls, 5-FU or FT-207.[37–39] The future work will carry on toxicology and

Table 7
IC_{50} value of **1** on tumor angiogenesis model.

Cell lines	IC_{50} (μM) of **1** to different assay				
	Proliferation	Migration	Tube formation[a]	VEGFR-2[b]	PDGFR-β[b]
HMEC-1	1.60 ± 0.48	0.05 ± 0.00	0.06 ± 0.02	–	–
NIH/3T3	–	–	–	2.20 ± 0.11	4.80 ± 0.30

[a] Tube-like structure formation of endothelial cells.
[b] Autophosphorylation level of tyrosine kinase receptors as the evaluating activity indicator.

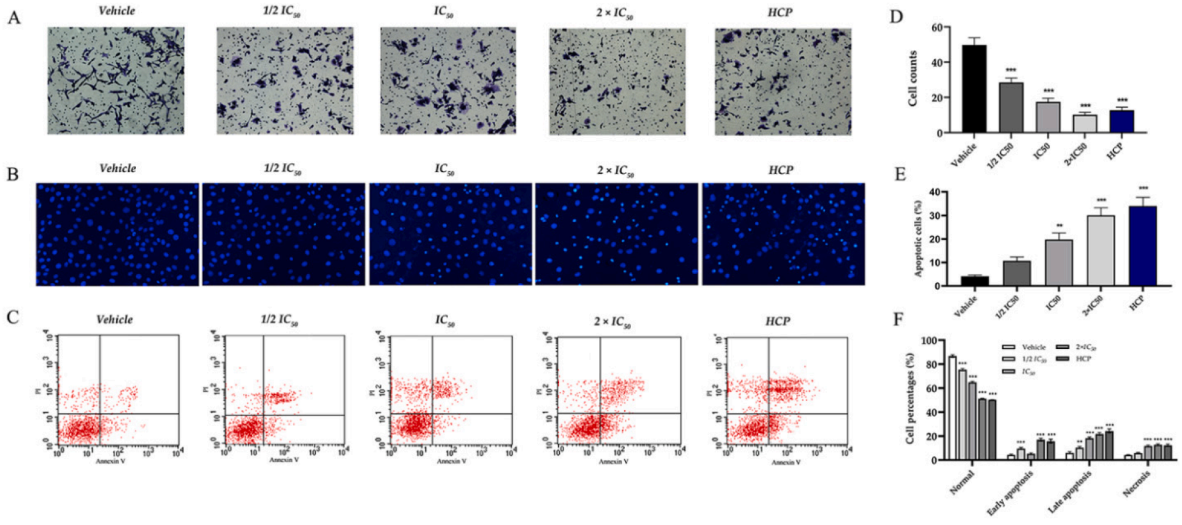

Fig. 4. Antitumor assays *in vitro* of coloquadranoside A (**1**) against HCT-116 cells. (A). Visual field map of Transwell assay; (B). Hoechst staining on HCT-116 among groups; (C). Annexin V and propidium iodide double-staining assay determined by flow cytometry; (D). Migration of HCT-116; (E) & (F). Apoptosis of HCT-116 among groups. Note: HCP, 10-hydroxycamptothecine, as pos. control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus vehicle, determined by one-way ANOVA or Brown-Forsythe and Welch ANOVA tests, n = 3.

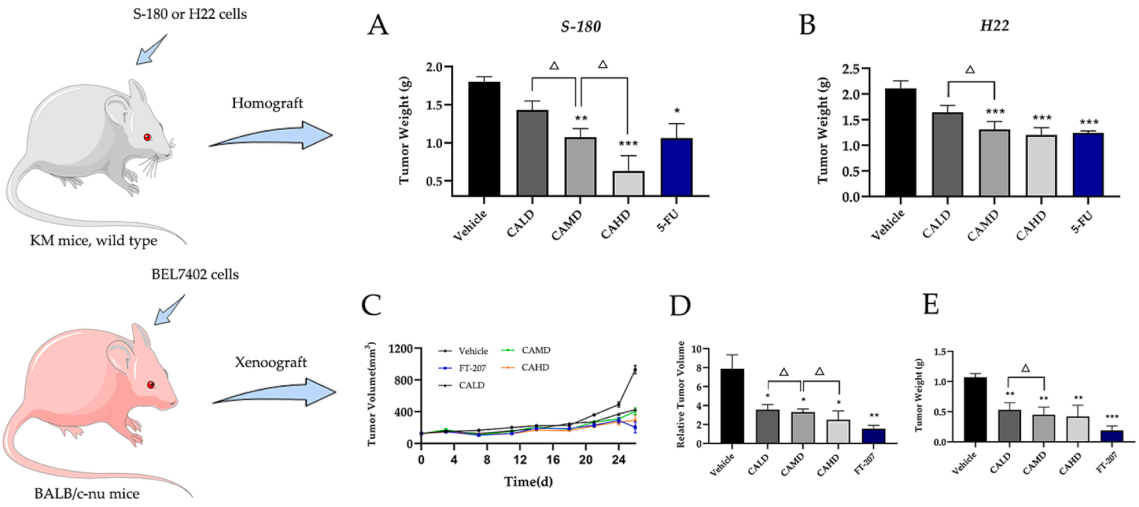

Fig. 5. Therapeutic effect *in vivo* of coloquadranoside A (**1**) against three mouse graft tumor models. Average tumor weight in wild type KM mice with the homograft model of S-180 sarcomas (A) or hepatocarcinoma cells H22 (B) at the last treatment day; the changing trend of tumor volume (C), average RTV (D), and average tumor weight at the last treatment day (E) in BALB/c-nu mice bearing BEL7402 xenograft tumors; CALD, CAMD and CAHD represent coloquadranoside A with low dose, middle dose and high dose, respectively; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus vehicle; $^{\triangle}$ $P < 0.05$, versus CAMD, determined by one-way ANOVA tests, n = 8.

Table 8
Effect of coloquadranoside A (**1**) on the growth of S-180, H22 and BEL7402 tumors (n = 8).

Group		Dosage (mg/kg)	TGI (%)	T/C (%)	*P*-value[a]
S-180	Vehicle	–	–	–	–
	1	0.5	21.6	–	>0.05
	1	5	44.2	–	<0.01
	1	50	55.5	–	<0.001
	5-Fu	25	39.4	–	<0.05
H22	Vehicle	–	–	–	–
	1	0.5	22.1	–	>0.05
	1	5	40.1	–	<0.001
	1	50	43.3	–	<0.001
	5-Fu	25	39.4	–	<0.001
BEL7402	Vehicle	–	–	–	–
	1	5	50.1	45.0	<0.05
	1	50	58.2	42.2	<0.05
	1	500	58.6	32.0	<0.05
	FT-207	100	81.8	20.0	<0.01

[a] *P*-value, compared with vehicle group, determined by one-way ANOVA test, using for evaluating whether TGI or T/C was valid or not.

pharmacodynamics to characterize its safety and optimal dose *in vivo*.

2.5. Effective immunomodulation in vivo

The immunosuppression of mice established by intraperitoneal injection of cyclophosphamide (CTX) is similar to that of human.[40,41] Our CTX model was successful in mice ($P < 0.05$, Fig. 6), confirmed by significantly lower carbon particle clearance index and the atrophy of spleen and thymus.[42–44] Meanwhile, the single most striking observation to emerge from the data comparison was that coloquadranoside A with the dose of 50–500 mg/kg significantly increased the clearance index and adjusted phagocytosis index in the immunosuppressed mice ($P < 0.05$, Fig. 6); in other words, it improved the clearance and phagocytosis function of mouse monocyte macrophages in immunosuppressed condition. Similarly, the injection of CTX induced lower thymus and spleen index and hemolysin level, which were significantly reversed by the treatment of coloquadranoside A of 50–500 mg/kg ($P < 0.001$; Fig. 6). It indicated that coloquadranoside A possesses the immunomodulatory activity in immunosuppressed mice.

Phagocytosis of mononuclear macrophages is noted as indicators reflecting the innate immune function, characterized by carbon particle clearance index.[42] The clearance and phagocytosis index of immunosuppressed mice was significantly increased by coloquadranoside A, which suggests its improvement of mononuclear immune function. These findings may help us to propose that coloquadranoside A enhances the innate immune system of immunosuppressed animals. Furthermore, the hemolysin experiment provided another substantive evidence of improvement of immune function. The hemolysin level, positively related to the adaptive immunity ability,[43] was significantly increased by coloquadranoside A in immunosuppressed mice. Its administration also improved the atrophy of spleen and thymus caused by CTX,[44] showing the potential for immunoenhancement in the immune organs. These evidences suggested that coloquadranoside A ameliorates the immune organ index, thereby advantageous to the enhancement of the humoral or adaptive immune function in immunosuppressed condition.

Together these results provided important insights into coloquadranoside A markedly improving both adaptive immunity and innate immunity of immunosuppressive mice, while the further researches are still needed for the specific mechanism of such considerable immunomodulatory activity *in vivo*.

3. Conclusions

As extracts of complex mixtures, triterpene glycosides are obtained in the multistage process of isolation and purification due to their low content and a large number of congeners and isomers. Ongoing chemical investigation and bioassay-guided isolation of the sea cucumber *C. quadrangularis* led to the characterization of compounds **1**–**5**, among which new coloquadranoside A (**1**) was found possessing a 16β-acetyloxy group and a Δ^{25} terminal double bond in the holostane aglycone with a 7(8)–double bond, and a sulfate group linked to C-4 of Xyl^{1} in the tetrasaccharide chain. The examined coloquadranoside A displayed the excellent antifungal, antitumor and immunomodulatory activities, and we first made a report of the improvement on innate and adaptive immune system under immunosuppression of such glycoside from the sea cucumber *C. quadrangularis*. Future work will be required to explore its exact activity mechanism, toxicology for safety, structure-activity relationship, and structural optimization. Anyway, the present finding provides evidence that this marine-derived sulfated triterpene glycoside may become an attractive candidate for further preclinical testing as a

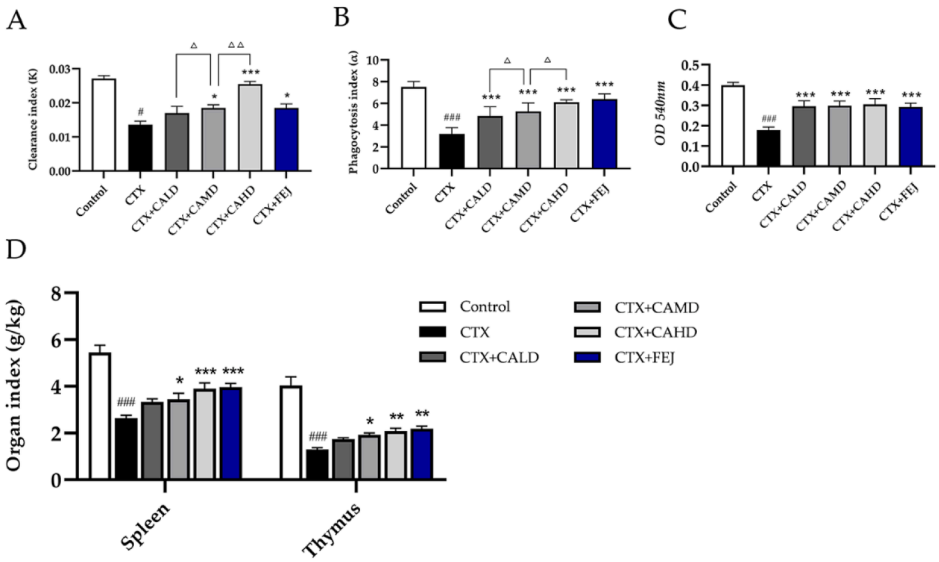

Fig. 6. The immunomodulatory activity *in vivo* of coloquadranoside A (**1**) in immunosuppressed mouse model. The carbon clearance index (A); Adjusted phagocytosis index (B); Hemolysin level (C), shown as OD_{540nm} value of serum; Immune organ index (D), g/kg; CALD, CAMD and CAHD represent coloquadranoside A with low dose, middle dose and high dose, respectively; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus CTX; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$, versus CTX + CAMD; determined by one-way ANOVA or Brown-Forsythe and Welch ANOVA tests, n = 8.

new drug probably.

4. Experimental section

4.1. General procedures

Melting points were determined on an XT5-XMT apparatus. Optical rotations were measured on a Perkin-Elmer-341 polarimeter. IR spectra were recorded on a Bruker Vector-22 infrared spectrometer. NMR spectra were recorded in C_5D_5N on a Bruker Avance-II-600 spectrometer with TMS as internal standard. ESI-MS was acquired on Q-TOF Micro LC-MS-MS mass spectrometer. GC–MS were performed on a Finnigan Voyager apparatus using a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm) with an initial temperature of 150 °C for 2 min and then temperature programming to 300 °C at a rate of 15 °C/min. HPLC was carried out on an Agilent 1100 liquid chromatograph equipped with a refractive index detector using a Zorba 300 SB-C18 column (250 × 9.4 mm i.d.). Column chromatographic separations were performed on silica gel H (200–300 mesh, 10–40 μm, Qingdao Marine Chemical Inc.; Qingdao, China), Lobar Lichroprep RP-C_{18} (40–63 μm; Merck) and Sephadex LH-20 (Pharmacia). Fractions were monitored by TLC on precoated silica gel $HSGF_{254}$ plates ($CHCl_3$-EtOAc-MeOH-H_2O, 4: 4: 2.5: 0.5) or RP-C_{18} (MeOH-H_2O, 1: 1), (Qingdao Marine Chemical Inc., Qingdao, China) and spots were visualized by heating Si gel plates sprayed with 15% H_2SO_4 in EtOH.

4.2. Reagents, controls and antibodies

Positive controls for antifungal assay like Itraconazole (ICZ), Terbinafine (TRB) and Fluconazole (FCZ) were supplied by Department of Pharmacology, School of Pharmacy, Second Military Medical University (SMMU), China, and 10-hydroxycamptothecin was provided by Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. Hoechst 33,258 and Annexin V-propidium iodide double-staining flowcytometry reagents were purchased from Sigma-Aldrich, Shanghai, China. Futraful (FT-207), 5-Fluorouracil (5-FU) and Fufang Ejiao Jiang (FEJ), as the positive drugs in the intracorporal screening. Cyclophosphamide (CTX) for injection, dissolved in normal saline to make 2.5 mg/mL solution immediately before using. Indian ink, diluted four times with normal saline. Filter and store in refrigerator at 4 °C. Potato medium, including 1.5% sucrose, 20.0% potato and 2.0% agar. Sabouraud liquid medium, including 1.0% peptone, 0.5% NaCl and 4.0% glucose. RPMI-1640 culture medium, product by Thermo Fisher Scientific Co., Ltd. DMEM was purchased from GIBCO, Life Technologies, Grand ISIAND, NY, USA. Sulforoxamine B (SRB) and the stimulator, VEGF and PDGF-BB, were purchased from Sigma-Aldrich Corporation, MO, USA. Trichloroacetic acid (TCA) and Tris base buffer were both analytical pure. Matrigel was purchased from Becton Dichinson, USA. Tyrosine phosphorylation monoclonal antibody PY-99, sheep anti mouse biotin labeled IgG (H + L) and Horseradish-Peroxidase-Streptavidin polymer (HRP-Avidin) were purchased from Vector, USA. The 10% complement was prepared from serum of guinea pigs with normal saline. Rabbit erythrocyte was taken from heart blood with fibrinogen removed, washing with normal saline to make 20% suspension.

4.3. Animal material

Specimens of *C. quadrangularis* was collected from the South China Sea near Guangdong province, China in May 2018. Taxonomy of the sea cucumber was identified by Prof. JR Fang, Fujian Institute of Oceanic Research, China. A voucher specimen (No. SA201842) was preserved in School of Medicine, Tongji University, Shanghai, China.

4.4. Extraction and isolation

The sea cucumber (5 kg, dry weight) was cut into pieces and extracted with 70% EtOH (20 L, each time for 1 h) under reflux. The EtOH extracts were combined and then evaporated. The residue was dissolved in H_2O (4 L). The H_2O-soluble fraction was passed through a DA101 resin column (60 × 30 cm; Nankai University, Tianjin, China), and eluted with dist·H_2O (until a negative reaction of chloride was observed) and 50% aq. EtOH. The combined 50% EtOH eluate was evaporated and subjected to Sepahedex LH-20 column (3 × 50 cm) equilibrated MeOH/H_2O (2:1). The fraction (12 g) containing saponins was chromatographed over silica gel column (dry column, 500 g, 2 × 50 cm, eluting with $CHCl_3$/MeOH/H_2O from 8:2:1 to 6.5:3.5:1, lower phase stepwise gradient) again. Each subfraction containing saponins was purified by semipreparative HPLC (Zorbax SB-C18, 5 μM; 250 mm × 9.4 mm i.d.; 58% aq. MeOH, 1.5 mL/min), and obtained pure **1** (32 mg; t_R 35.89 min).

*Coloquadranoside A (**1**)*. White amorphous powder; mp 223–226 °C; $[\alpha]_D^{20}$ = -16.7 (*c* = 0.60, pyridine); IR (KBr): υ_{max} 3419, 1747, 1647, 1233, 1039 cm^{-1}; ^{1}H- and ^{13}C NMR: shown in Tables 1 and 2; ESI-MS: *m/z* 1223 $[M+Na]^+$, *m/z* 1177 $[M-Na]^-$ (calc. for $C_{55}H_{85}Na_2O_{25}S^+$, 1223).

4.5. Acid hydrolysis of coloquadranoside A (1)

Compound **1** (1 mg) was heated in an ampule with 2 M CF_3COOH (1 mL) at 120 °C for 2 h. The reaction mixture was evaporated to dryness, and the residue was partitioned between CH_2Cl_2 and H_2O. The aqueous phase was evaporated under reduced pressure. Then, pyridine (1 mL) and $NH_2OH{\cdot}HCl$ (2 mg) were added to the dried residue, and the mixture was stirred at 90 °C for 30 min. After that time, 1 mL of Ac_2O was added, and the heating at 90 °C was continued for another hour. The soln. was concentrated, and the resulting aldononitrile peracetates were analyzed by GC–MS. The carbohydrates were determined by comparing the retention times and MS behavior with standard aldononitrile peracetates (Sigma) prepared from authentic sugars by the same procedure performed for the sample. D-xylose, D-quinovose, and 3-*O*-methyl-D-glucose were identified in a ratio of 2: 1: 1.

4.6. Desulfation of coloquadranoside A(1)

Compound **1** (10 mg) was dissolved in pyridine/dioxane (1:1, 3.0 mL) and heated under reflux for 4 h. The mixture was partitioned between H_2O and BuOH. The BuOH extract was evaporated and the residue purified by reversed-phase HPLC (Zorbax-300-SB-C_{18}, 80% MeOH/H_2O) at a rate of 1.5 mL/min to yield the pure **1a** (6 mg).

4.7. Strains, cell lines and mice

The strain, *Pyricularia oryzae* P-2B, was provided by the University of Tokyo, Japan. The fungus strains *Candida albicans* ATTCC76615, *Cryptococcus neoformans* ATTCC32609 were provided by Changzheng hospital, SMMU, Shanghai, China; *Candida tropicalis* (clinical strain) and *Candida parapsilosis* (clinical strain) were provided by Changhai hospital, SMMU, Shanghai, China. Cancer cell lines, human lung cancer (A-549), human gastric cancer (MKN-28), human breast cancer (MCF-7), human colon cancer (HCT-116), human hepatocellular carcinoma (BEL-7402), human ovarian cancer (HO-8910), human promyelocytic leukemia (HL-60), human lung adenocarcinoma (SPC-A4), mouse sarcoma (S-180) and mouse hepatocarcinoma (H22), and human microvascular endothelial cell line, HMEC-1, were provided by Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. Mouse Fibroblast (NIH/3T3) was purchased from American Type Culture Collection, Manassas, VA, USA, and its highly expressed lines of VEGFR-2 and PDGFR-β was obtained by plasmid transfection. BALB/c-nu mice, SPF grade, provided by Chinese Academy of Medical Sciences, Beijing, China, while KM mice, wild type, cleaning grade, provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China, and all procedures were manipulated by the protocols including the animal care, and approved by Laboratory Animal Research Center, Tongji University, Shanghai, China. The cancer lines were maintained in RPMI-1640 complete medium in a humidified 5% CO_2 atmosphere at 37 °C. RPMI-1640 complete medium was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM β-mercaptoethanol, penicillin (100 U/mL) and streptomycin (100 μg/mL).

4.8. Antifungal and cytotoxicity tests in vitro

The antifungal activity of the compounds was tested against four strains, *C. albicans* (ATCC76615), *C. neoformans* (ATCC32609), *C. parapsilosis* (clinical strain) and *C. tropicalis* (clinical strain). The data of antifungal activity was evaluated by measuring optical density (OD) at 630 nm using an automatic microplate reader.[31,45] MIC_{80} was defined as the first well with an approximate 80% reduction in growth compared with the growth of the drug-free-well. The data represents the means of three independent experiments in which the compound concentration was tested in three replicate wells. Itraconazole (ICZ), Terbinafine (TBNF) and Fluconazole (FCZ) were used as positive controls. The cytotoxicity of compounds against the eight human cancer cell lines, A-549, MKN-28, MCF-7, HCT-116, BEL-7402, HO-8910, HL-60, SPC-A4, was evaluated by SRB assay as described.[30] The anticancer agent 10-hydroxycamptothecin served as a positive control. The activity of compounds was determined at 100, 10, 1, 0.1, and 0.01 μM (each concentration being tested in triplicate). Data were calculated as percent inhibition (I) according to the formula: $I = (OD_C - OD_T)/OD_C \times 100\%$, where OD_T and OD_C are the mean optical densities of the treatment of compounds and the solvent control, respectively. The concentration inducing 50% inhibition of cell growth (IC_{50}) was determined graphically for each experiment by curve fitting using GraphPad Prism (ver. 8.0, GraphPad Software Inc., San Diego, CA, USA), and derived by DeLean et al.,[46] the equation was calculated by Logit method. IC_{50} values represent the mean of three independent experiments.

4.9. Antitumor and tumor angiogenesis in vitro

Minimum morphological deformation concentration (MMDC) of coloquadranoside A, as described,[31,32] was tested against *P. oryzae*. HCT-116 cells were seeded for antitumor assay, and following coloquadranoside A (0, 1/2 IC_{50}, IC_{50} and two-fold of IC_{50}, respectively) or HCP as positive control solution were added into the cells and incubated. Then the migration assay was carried out by routine procedure of Transwell. Each insert was imaged in for five random fields at 100 × magnification, and the analysis was done using ImageJ program (ver. 1.48, National Institutes of Health, Bethesda, MD, USA). As for the Hoechst assay, briefly, the cells were stained with Hoechst 33,258 staining solution, and the nuclear morphology was examined using an inverted fluorescence microscope (Leica DMI4000B, Germany, × 400). Cells were counted from five random fields and the number of apoptotic cells was expressed as a percentage (%) of the total number of counted cells. Besides, the cells for apoptosis assay were collected after administration. They were stained with Annexin V-FITC (5 μL) and propidium iodide (PI; 5 μL) afterwards in the dark at 4 °C. Immediately examine with a flow cytometer equipped with FACS Comp software (BD Biosciences, Franklin Lakes, NJ, USA). While HMEC-1 cells were also seeded as usual, then the drugs were added. The proliferation and migration assays were carried out by routine procedure of SRB method or Transwell, then IC_{50} values were calculated. As for tube-formation assay, matrigel was quickly added to the plate, allowed to stand to make the glue solidify. Seed cells and add drugs as above. Culture, then the total length of tube was measured by Adobe Photoshop to calculate IC_{50} values. Additionally, NIH-3 T3 with high VEGFR-2 or PDGFR-β expression were seeded for phosphorylation assay. The cells were starved for 24 h before the drugs added. They subsequently were stimulated with VEGF (500 ng/mL) or PDGF-BB (150 ng/mL). Fix with 4% paraformaldehyde, and seal the culture plates with TPBS. Add PY-99, IgG (H + L), and HRP-Avidin in turn. The OD was determined at 405 nm after the reaction of ATBS. Calculate IC_{50} to characterize the phosphorylation level of VEGFR-2 or PDGFR-β. Three independent experiments were carried out for each group in these assays *in vitro*.

4.10. Mouse homograft and xenograft models

S-180, H22 and BEL7402 tumor lines were cultured in the usual way. Select the former two to assay the therapeutic effect of compound **1** in the homograft tumor mice. The tumor lines were made into cell suspension respectively, inoculated subcutaneously in the right axilla of the wild-type KM mice, about 2.5×10^6 cells/animal. After 24 h, the mice were randomly divided into different groups (Table 8), administrated with different dose drugs intragastrically for 7 days. Sacrifice the mice 24 h after the discontinuation of administration. The average tumor weight and tumor growth inhibition rate (TGI) would be as evaluation, the latter calculated by $TGI = (W_C - W_T) / W_C \times 100\%$, where W_T, the average tumor weight of the administration groups; W_C, the average tumor weight of the control group; the effective could be proved by TGI > 30% and $P < 0.05$. BEL7402 lines were undertaken to the continuing

therapeutic assay. The xenograft model of bearing BEL7402 BALB/c-nu was established as the above method. After inoculation, the tumor volume (TV) was measured with vernier caliper at regular intervals, calculated by $TV = 1/2 \times a \times b^2$, where a & b represent the long and the short diameter of tumors. When TV approximately 100–200 mm^3, administrate intragastrically once a day for 25 days. Measure TV and weight of each group twice a week. The mice were sacrificed as above. The tumors were weighed and measured, and TGI, relative tumor volume (RTV), relative tumor proliferation rate (T/C) and other evaluation were calculated, while $RTV = V_t / V_0$, and $T/C = RTV_T / RTV_C \times 100\%$, where V_0, the tumor volume measured at the time of grouping (day 0); V_t, the tumor volume measured at each time (day t); when $T/C \leq 60\%$ and $P < 0.05$, valid; otherwise, invalid.

4.11. Immunomodulatory assay in vivo

KM mice were randomly divided into different groups. The treatment groups were intragastrically administrated with FEJ (10 mL/kg) or different concentrations of coloquadranoside A (500 mg/kg, 50 mg/kg and 5 mg/kg), once a day for 14 consecutive days, while the control group and the model group were administered with 0.5% sodium carboxymethyl cellulose with equal volume. On the 7th, 9th and 11th day, except for the control group, mice in each group were injected with CTX 50 mg/kg intraperitoneally, 25% (*v/v*) Indian ink injected into tail vein with 0.1 mL/10 g one hour after the last administration. Two and ten minutes after ink injection, 20 μL orbital venous plexus blood was respectively collected and added to 2 mL distilled water. The OD value of the samples was measured at 680 nm wavelength, and the carbon particle clearance index (K) and adjusted phagocytosis index (α) were calculated, $K = (lgOD_1 - lgOD_2) / (t_2 - t_1)$, and $\alpha = K^{1/3} \times W_{body} / (W_{liver} + W_{spleen})$. Additionally, the mice were also divided into different groups as above, and after five-time administration, the groups were immunized by intraperitoneal injection of 20% rabbit erythrocyte saline 0.2 mL except for the control. Then, the treatments of drugs were continued for 8 days. On the 2nd, 4th and 6th day after immunization, the mice were injected with CTX 30 mg/kg intraperitoneally to establish the model except for the control. An hour after the last administration, the blood was taken from eyeballs to prepare serum. At the same time, mice were dissected, spleen and thymus weighed. The serum was diluted hundredfold with normal saline. Then, take 1 mL serum to mix with 20% rabbit erythrocyte suspension 0.5 mL and 10% complement 0.5 mL. Keep them in 37 °C water bath for 30 min. The reaction was terminated at 0 °C. The supernatant's OD value at 540 nm, having positive correlation with serum hemolysin level, was measured to characterize the different levels among groups thereby.

4.12. Statistical analysis

The experimental data was expressed by (mean ± SD). Statistical significance was assessed by SPSS program (ver. 25.0, SPSS Inc., Chicago, IL, USA). Student's *t* test or one-way ANOVA with Dunnett's test was used to perform the significance test, while Brown-Forsythe and Welch ANOVA tests would be used when missing variance homogeneity. $P < 0.05$ was considered statistically significant. The data in bioactivities complies with the recommendations on experimental design and analysis in pharmacology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116188.

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