Hindawi Publishing Corporation
Journal of Chemistry
Volume 2014, Article ID 525141, 9 pages
http://dx.doi.org/10.1155/2014/525141

Research Article

Purification and Characterization of Bioactive Compounds from *Styela clava*

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Received 17 March 2014; Accepted 1 April 2014; Published 30 April 2014

Academic Editor: Qiusheng Zheng

The immunomodulatory activity of extract from *Styela clava* was studied systematically based on activity tracking *in vitro* in order to find out novel-structured secondary metabolite. The proliferation rates of mouse splenic lymphocytes and peritoneal macrophages were used as screening index, as well as NO release promoting activities. The crude extract (CE) and its different polar fractions from *S. clava* all exhibited proliferative activity of splenolymphocytes and mouse macrophages, as well as NO release promoting activities, among which petroleum ether fraction (PE) showed the strongest effect. The antioxidant experiment *in vitro* showed that CE demonstrated antioxidant ability in 1,1-diphenyl-2-picrylhydrazyl (DPPH) system and the beta carotene-linoleic acid system; the activity of ethyl acetate fraction (ET) was much stronger than that of the others. Further isolated by silica gel column chromatography, ET was classified into seven sub-components (E1~E7) listed in the order of activity as E5 > E6 > E4 > E3 > E7 > E2 > E1. Five compounds were separated as (1) cholesteric-7-en-3β-ol, (2) cholesteric-4-en-3β,6β-diol, (3) cholesterol, (4) batilol, and (5) ceramide, among which (1), (2), and (4) were isolated for the first time from *S. clava*.

1. Introduction

The ascidians are commonly found in waters all over the world, along the coasts and deep to the bottoms, which is the most important marine source for active agents except sponge. In the early 1970s, it had been found that the extract from ascidians had a variety of bioactivities such as cytotoxicity, antitumor *in vivo*, and immune regulation. Since 1980s, a greater variety of bioactive substances were extracted from the ascidians with the activities of antitumor, antivirus, antimicrobial, immune regulation, and biocatalysis. Further studies showed that those active chemical compounds in sea squirt mainly included peptides, alkaloids, polyethers, macrolides, terpenes, and polysulfides [1–9], among which some have been tested at the clinical phase as anticancer reagents [10–13].

By strengthening organisms' defense and host immune system against tumor cells, cell immunity can improve its overall antitumor ability to inhibit tumor and thus play an important role in immune antitumor treatment. Alcohol from *Styela clava* (5α,8α-cyclicobioxygen-24-bimethyl-6-vinyl-3β-cholesterol, SC), a lead compound against a variety of viruses, has a wide application to immune regulation as a supplement to maintain normal immune response for the removal of viruses and recovery [14].

The ascidian has become the hot topic for research and development both at home and abroad for its numerous active agents with high activity. *Styela clava*, the dominant population in the Yellow Sea and the Bohai Sea, has a negative impact on marine culture for its wide distribution and large quantity while the changes in its population may be regarded as an index for environment contamination. If fed in sea water in proper conditions, *S. clava* may grow and reproduce very fast with low cost; therefore, with rational exploitation, *S. clava* can become treasures instead of waste to benefit both economy and environment protection.

S. clava remains neglected as a potential marine chemical resource, and there are few reports on its components

worldwide besides few individual components without in-depth investigation. Up to now, there have been no reports of the systemic in-depth study on *S. clava*'s compounds and their activity. Integrated exploitation of its active agents may on one hand provide theoretical basis for resource exploitation to achieve maximum economic results and on the other improve our lives and medical care to achieve direct social results.

This paper is a study on *S. clava* in terms of its chemical structure and bioactivity to obtain novel-structured active agents as a theoretical basis for new drugs or lead compound for new drugs. These studies may be used as references for the research and development of marine functional food and drugs.

2. Materials and Methods

2.1. Materials. *Styela clava* was collected from Yantai Sishiliwan Bay, Shandong Province, China, in August 2010; adherents were removed and frozen.

Kunming mice of clean grade were purchased from Shandong Animal Experiment Center, with weight being 18–22 g for each.

ConA, LPS, MTT, peptone, 1,1-DPPH, β-carotene, and linoleic acid were purchased from Sigma Chemical Co. (USA), RPMI1640 from Gibco, fetal bovine serum from HyClone, DMSO from AMRESCO, and penicillin and streptomycin from Shandong Lukang Pharmaceutical Group Co., Ltd. Other analytical reagents were purchased from home markets.

2.2. Methods

2.2.1. Extraction of Active Agents from S. clava. Tissue of *S. clava* (15 kg) was minced, centrifuged, and then soaked in 95% ethanol three times in volume for 1 week at room temperature. The extraction was repeated three times. The combined extract was concentrated under vacuum to give a crude extract (CE) as brown extractum of 205 g. The crude extract was suspended in distilled water of 2000 mL and then further extracted with petroleum ether, chloroform, ethyl acetate, and butanol (800 mL ×3), respectively, resulting in petroleum ether extract (PE) 78.7 g, ethyl acetate extract (ET) 18.6 g, butyl alcohol extract (BU) 40.2 g, and the remainder (AR) 64.7 g.

2.2.2. Isolation, Purification, and Structural Identification of the Chemical Components from S. clava. After activity screening, normal phase silica gel column chromatography (CC) and ODS reversed phase silica gel column chromatography were adopted, respectively, for the most active ET, while petroleum ether and acetone, chloroform and carbinol were used for silica gel column chromatography gradient elution. Seven components (E1–E7) were isolated, among which components E2, E3, E4, and E5 for their large quantity were further purified by Sephadex LH-20, PTLC, Amberlite XAD-2, and recrystallization and 5 compounds were obtained and their structures were identified by IR (Nicolet impact 400), MS (HP 5988A GC/MS), and 1D NMR and 2D NMR (Bruker-500 MHz-FT-NMR).

2.2.3. Proliferation of Spleen Lymphocyte. Ten days after vaccinated S180, Kunming mice of clean grade were killed by cervical dislocation and spleens were taken out under asepsis for the preparation of lymphocyte suspension which was incubated in the 96-well plates (Coastar, USA) at the concentration of 1×10^7 cells/mL with 100 μL for each well and six repeats for each treating. Medium of 100 μL was added to the control group, 10 μg/mL ConA to the positive group and the sample solutions of different concentration to the experiment groups resulting in the concentrations of 12.5, 25, 50, 100 and 200 μg/mL, cultured at 37°C in 5% CO_2 incubators (by SHEL LAB) for 44 h, and, afterwards, 5 mg/mL of MTT was added with 20 μL for each well and further cultured for 4 h. After the media were removed, 0.15 mL of DMSO was added to each well and shaken for 10 min for complete mixture at room temperature for 10 min before ELX-800 (BioTek Instruments Inc.) was used to measure absorbance at 570 nm. Consider

$$\text{Proliferation ratio of lymphocytes (\%)} = \left[\frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}}\right] \times 100\%, \quad (1)$$

where A_{sample} is the absorbance of tested sample and A_{control} is the absorbance of control group.

2.2.4. Proliferation of Macrophages. 2% (w/v) peptone of 2 mL was injected into the mice's peritoneal cavity every day for three days. After cervical dislocation, precooled Hank's of 5 mL was injected into the peritoneal cavity; then abdominal fluid was sucked and the supernatant was removed by centrifugation at 2000 rpm; the precipitate cells were washed twice.

1×10^6/mL of cell suspension was made with 10% of calf serum in RPMI 1640, incubated in the tissue culture plate with 100 μL for each well and cultured at 37°C in 5% CO_2 incubators for 2 h. The upper medium was removed and the wells were washed with PBS twice to remove the nonadherent cells; so macrophage monolayers were obtained. Fresh medium was put into the 96-well plates, following with fresh medium of 100 μL to the control group, 100 μL LPS to the positive group (with final concentration of 2 μg/mL), and 100 μL different samples of different concentrations to the other groups (the sample concentrations are as in Section 2.2.3), and cultured at 37°C in 5% CO_2 incubators for 24 h. Afterwards, upper medium was removed and new medium was added with 100 μL for each hole as well as 5 mg/mL of MTT with 20 μL, further cultured for 4 h; then the supernatant was carefully sucked and 150 μL DMSO was added before shaking for 10 min for complete mixture. After

placing at room temperature for 10 min, ELX-800 was used to measure absorbance at 570 nm. Consider

$$\text{Proliferation ratio of macrophages (\%)} = \left[\frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}}\right] \times 100\%, \quad (2)$$

where A_{sample} is the absorbance of tested sample and A_{control} is the absorbance of control group.

2.2.5. NO Release Activities of Macrophages. Macrophages were prepared with the same method as in Section 2.2.4.

2.5×10^5/mL of cell suspension was made with 10% of calf serum in RPMI 1640, in sterile conditions inoculated on the tissue culture plate with 100 μL for each well and three wells for each sample were cultured at 37°C in 5% CO_2 incubators for 2 h. Then LPS solution (with final concentration of 2 μg/mL) of 100 μL was put into the positive group, medium 1640 of 100 μL was added to the control group, and 100 μL with different samples of different concentrations was added to the other groups, and further cultured for 48 h. Afterwards, upper medium of 50 μL from each well was removed and transferred to a new 96-well plate and measured according to the following method.

Standard Curve. Standard solution nitrite of 0.1 mol/L was diluted in 1 : 1000 with medium. A1~H3 on the tissue culture plate were used for standard curve. Standard solution nitrite of 100 μL was put into A1~A3, respectively, and medium of 50 μL was put into B1-H3, respectively. Doubling dilution was conducted to A–G in turn and the final fluid from G was removed and nitrite concentration coefficient (100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 μmol/L) was obtained. Standard curve was necessary before each test with the same medium as for the samples.

Measurement. NED solution and sulfanilamide solution were kept at room temperature for 15 to 30 min. Each of tested samples of 100 μL was placed on the tissue culture plate, 2 to 3 repeated wells were used for every sample. Sulfanilamide solution of 50 μL was put into all standard curve wells and sample wells were kept in dark place at room temperature for 5 to 10 min and then the same was done with NED solution of 50 μL. Absorbance at 540 nm was measured within 30 min and the nitrite concentration in the samples was calculated according to standard curve.

2.2.6. In Vitro Antioxidant Activity

(1) DPPH Radical Scavenging Assay. Modified Binsan's method [15] was adopted. 1.5 mL sample in different concentrations was added to 1.5 mL of 0.2 mM DPPH in ethanol.

1.5 mL DPPH of 0.2 mmol in anhydrous ethanol was added to all the samples of 1.5 mL, after mixing vigorously, and kept at room temperature in dark for 30 min. The absorbance of the resulting solution was measured at 517 nm with water as the control and the ability to scavenge the DPPH radical was expressed by clearance rate (E) in the following formula:

$$E\,(\%) = \left[1 - \frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{control}}}\right] \times 100\%, \quad (3)$$

where A_{sample} means the absorbance of test sample solution (DPPH + sample), $A_{\text{sample blank}}$ means the absorbance of the sample only (ethanol + sample), and A_{control} means the absorbance of control (DPPH + ethanol).

The half clean concentration (EC_{50}) is calculated by regression equation of E and sample concentration.

(2) β-Carotene-Linoleic Acid System. Modified Jayaprakasha's method [16] was adopted. β-carotene of 0.2 mg was dissolved in 0.2 mL trichloromethane, into which linoleic acid of 20 mg and Tween-40 of 200 mg were added and mixed completely. After chloroform was removed under vacuum at 40°C, distilled water of 50 mL (oxygenated for 2-3 min) was added. The reaction emulsion was prepared after vigorous agitation. The control was prepared as above except for the addition of β-carotene. Sample solution of 50 μL and reaction medium of 200 μL were added into 96-well plates, respectively, and the absorbance was measured at 450 nm immediately ($t = 0$), and remeasured every 20 min until the color of β-carotene faded in the control (about 240 min). Three parallel measurements were conducted and the antioxidant activity (AA) was expressed in the following formula:

$$\text{AA} = \left[1 - \frac{A_0 - A_t}{A_0' - A_t'}\right] \times 100, \quad (4)$$

where A_0 and A_t were the absorbance of the sample, while $t = 0$ and $t = 240$ min, respectively, and A_0' and A_t' were the absorbance of the control, while $t = 0$ and $t = 240$ min, respectively.

(3) Reducing Power. The reducing power was determined according to Yen and Duh's method [17]. All samples were dissolved with phosphate buffer solution (0.2 mol/L, pH 6.6) of 2.5 mL, and 1% potassium ferricyanide (w/v) of 2.5 mL was added before reacting at 50°C for 20 min; then 10% trichloroacetic acid (w/v) of 2.5 mL was added to stop reaction. Reaction mixture was centrifuged at 650 ×g for 10 min; supernatant of 2.5 mL was mixed with distilled water of 2.5 mL and 0.1% $FeCl_3$(w/v) of 0.5 mL. The absorbance was measured at 700 nm and the higher absorbance means the stronger reducing power. The reducing power is converted in ascorbic acid and expressed as ascorbic acid equivalent (AscAE) in milligrammes of ascorbic acid per gramme of sample (AscAE: mg/g AscA).

2.3. Statistical Analysis. All data were expressed by mean ± standard deviation. All statistical analyses were carried out using SPSS 13.0 for Windows. Tukey's multiple comparison tests were used to detect differences between groups, and a probability of $P < 0.05$ was taken as an acceptable level of significance.

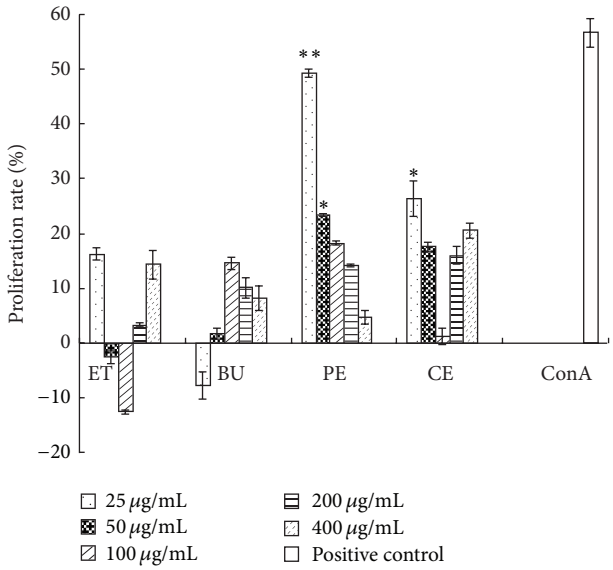

Figure 1: Effects of extracts on the proliferation of the mouse splenocytes. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control.

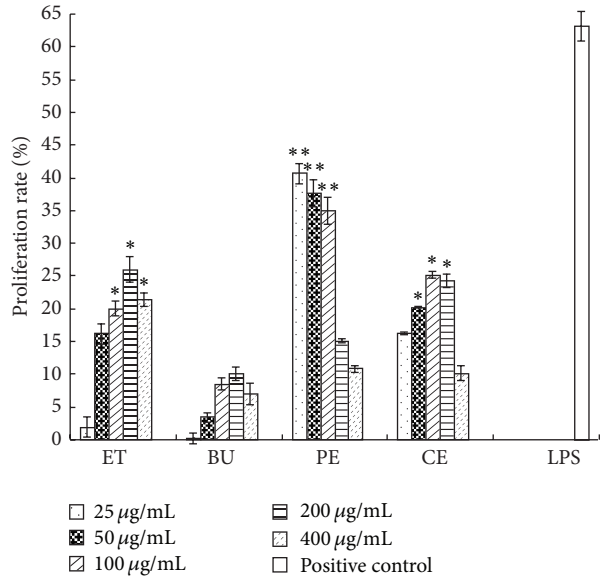

Figure 2: Effects of extracts on the proliferation of the mouse peritoneal macrophages. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control.

3. Results and Discussion

3.1. Proliferation of Spleen Lymphocyte. As organism's first line defending tumor, immune system plays an important role in the recognition and removal of malignant cells. The immune status evaluation of the body bearing neoplasm is important for mechanism study of antitumor drugs.

As shown in Figure 1, four extracts of *S. clava* demonstrate significant activity on splenocytes proliferation (compared with the control, $P < 0.05$, or $P < 0.01$), among which the proliferation effect of PE is the most active, with proliferation rate being near 50% at a concentration of 25 μg/mL, close to the positive control group ConA (56.71%). However, its proliferation rate reduces with the increasing concentration, and the proliferation rate is only 4.76% when the concentration reaches 400 μg/mL, which shows that PE inhibits the proliferation of the mouse splenocytes at high concentration. CE is less active than PE on the proliferation of the mouse splenocytes, demonstrating a rising trend at first and then a falling one. The effects of ET and BU are relatively weak, and ET shows distinctive inhibition at 50 μg/mL and 100 μg/mL with the inhibition rate of 12.62% at 100 μg/mL, but, on the contrary, BU reaches the strongest level at this concentration with the proliferation rate of 14.52%, while distinctive inhibition was shown below this point.

3.2. Proliferation of Macrophages. Figure 2 shows the effects of *S. clava*'s extracts on the proliferation of the mouse peritoneal macrophages. The four extracts all show significant effect on the proliferation of the mouse macrophages at all designed concentrations (compared with the control, $P < 0.05$ or $P < 0.01$), but it is weaker than that of the positive control LPS as proliferation rate of 63.19%. PE is the most active extract on the proliferation at concentration of 25 μg/mL, with proliferation rate 40.70%, but, afterwards, the proliferation rate drops with the increasing concentration and the proliferation rate is only 10.85% when the concentration reaches 400 μg/mL, showing similar trend as on the lymphocytes.

ET and CE are close on the effect of proliferation. ET reaches the lowest point at 25 μg/mL with the proliferation rate of 1.94% and the highest point at 200 μg/mL with the proliferation rate of 25.97%. CE shows similar trend as ET, reaching the highest and lowest points at 100 μg/mL and 400 μg/mL with the proliferation rate of 25.19% and 10.17%, respectively. BU shows the weakest effect, with the highest proliferation rate of only 10.08% at 200 μg/mL and the same effect as the control at 25 μg/mL; the proliferation rates at all the other concentrations are all below 10%.

3.3. NO Release Activities of Macrophages. Macrophages are the important components in the body immune system, functioning as immunization effect regulating the immune system, and NO is one of the active agents produced by macrophages, after they are activated [18]. Figure 3 shows effects of extract from *S. clava* on the NO release activities of mouse macrophages. The effects of ET, PE, and CE are all in positive correlation with concentration in promoting the release of NO. PE shows the strongest effect among them, reaching the highest point of 16.14 μmol/mL at 400 μg/mL. CE is less effective with NO releasing the highest point of 14.76 μmol/mL at 400 μg/mL. The effects of BU and ET are rather weak, only 8.70 μmol/mL for ET at 400 μg/mL as the highest point, while, at the other concentrations, the NO releasing abilities for ET and BU are close to and approaching 6 μmol/mL.

Table 1: DPPH scavenging activity of different components isolated from *S. clava*.

Component	DPPH scavenging rate (%)			
	50 (μg/mL)	100 (μg/mL)	150 (μg/mL)	200 (μg/mL)
CE	19.2 ± 0.37	30.12 ± 0.60	37.9 ± 0.64	42.6 ± 0.88
PE	4.83 ± 0.11	10.11 ± 0.22	14.24 ± 0.38	18.50 ± 0.37
ET	22.90 ± 0.70	38.10 ± 1.14	47.48 ± 1.30	50.64 ± 1.12
BU	8.38 ± 0.21	19.29 ± 0.37	25.32 ± 0.49	30.77 ± 0.62
AR	2.10 ± 0.05	3.05 ± 0.11	4.26 ± 0.08	4.89 ± 0.10

Table 2: DPPH scavenging activity of subcomponents E1–E7 isolated from ET of *S. clava*.

Subcomponent	DPPH scavenging rate (%)			
	50 (μg/mL)	100 (μg/mL)	150 (μg/mL)	200 (μg/mL)
ET	22.90 ± 0.70	38.10 ± 1.14	47.48 ± 1.30	50.64 ± 1.12
E1	8.24 ± 0.26	14.17 ± 0.40	18.64 ± 0.59	20.80 ± 0.65
E2	11.37 ± 0.38	18.19 ± 0.88	24.30 ± 1.62	29.18 ± 1.25
E3	18.46 ± 0.64	25.30 ± 1.24	34.28 ± 1.22	45.80 ± 0.99
E4	22.80 ± 0.32	38.20 ± 1.02	49.37 ± 1.57	56.78 ± 1.01
E5	30.26 ± 0.88	46.20 ± 0.92	59.16 ± 1.11	67.31 ± 1.65
E6	24.92 ± 0.77	42.00 ± 1.06	53.40 ± 0.70	64.42 ± 1.62
E7	14.00 ± 0.31	20.29 ± 0.66	27.13 ± 0.48	32.50 ± 1.01

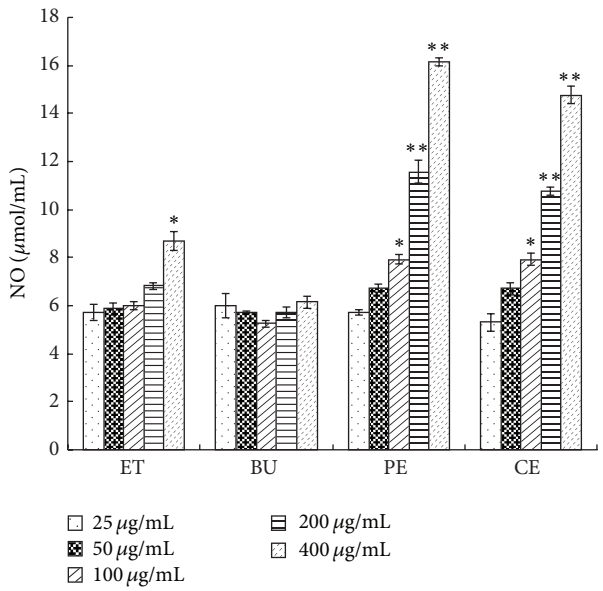

Figure 3: Effects of extracts on NO production. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control.

3.4. In Vitro Antioxidant Activity

3.4.1. DPPH Radical Scavenging Assay. The DPPH free radicals scavenging method is a colorimetric assay and can be used to evaluate the radical scavenging activity in a short time. DPPH is an organic free radical with maximal absorption at 517 nm. When with free radical scavenger, DPPH's lone electron will be paired and its absorbance will decrease, which can be used to evaluate its antioxidant activity. As shown in Table 1, there was a positive correlation between DPPH scavenging activity of CE and its concentration. Among the four extracted components, PE and BU demonstrate poor scavenging activity, while AR shows no scavenging activity. The scavenging activity of ET reaches 50.64% at 200 μg/mL, much higher than CE ($P < 0.05$), indicating that compounds with DPPH scavenging activity in *S. clava* are mostly medium polar.

E1–E7 subcomponents are isolated from ET with silica gel column chromatography, whose DPPH scavenging activity is shown in Table 2. At all experiment concentrations, E5 shows the strongest scavenging activity followed by E6, E4, E3, E7, and E2 with E1 the weakest. E5 and E6 show much stronger scavenging activity than ET ($P < 0.05$), while E4 is close to ET in scavenging activity. EC_{50} of E5 and E6 are 122.06 μg/mL and 139.68 μg/mL, respectively.

3.4.2. Antioxidant Activity in β-Carotene-Linoleic Acid System. β-carotene is a polyene pigment apt to be oxidized to fade. In the reaction medium, the superoxide produced by the oxidized linoleic acid results in β-carotene's fading, and, as time goes on, the absorbance decreases. The degree of β-carotene's fading depends on the antioxidant activity in the system. Table 3 shows antioxidant activities of different components isolated from *S. clava* in the β-carotene-linoleic acid system. CE has some antioxidation activities, positively correlated with its concentration. ET is much higher in its antioxidant activity than other components, reaching 50% at 200 μg/mL and is even higher than the positive control AscA at all experimental concentrations, which further indicates that compounds with higher antioxidant activity in the β-carotene-linoleic acid system are mostly medium-polar components.

Table 3: Antioxidant activity of different components isolated from *S. clava* in the β-carotene-linoleic acid system.

Component	Antioxidant activity (%)			
	10 (μg/mL)	50 (μg/mL)	100 (μg/mL)	200 (μg/mL)
CE	10.62 ± 0.38	20.10 ± 1.12	29.48 ± 1.30	40.35 ± 1.12
PE	4.60 ± 0.21	10.22 ± 0.44	16.50 ± 0.49	22.40 ± 0.68
ET	12.70 ± 0.65	28.76 ± 1.12	37.42 ± 0.88	48.37 ± 1.55
BU	7.20 ± 0.48	14.39 ± 0.62	22.60 ± 0.77	26.00 ± 0.89
AR	2.15 ± 0.05	6.90 ± 0.14	8.20 ± 0.18	9.80 ± 0.11
AscA	10.50 ± 0.12	20.70 ± 0.31	32.90 ± 0.18	46.60 ± 0.32

Table 4: Antioxidant activity of subcomponents E1–E7 isolated from ET in the β-carotene-linoleic acid system.

Subcomponent	Antioxidant activity (%)			
	10 (μg/mL)	50 (μg/mL)	100 (μg/mL)	200 (μg/mL)
ET	12.70 ± 0.65	28.76 ± 1.12	37.42 ± 0.88	48.37 ± 1.55
E1	6.46 ± 0.50	13.80 ± 0.46	28.20 ± 0.64	22.19 ± 0.97
E2	7.42 ± 0.31	15.40 ± 0.74	24.74 ± 1.07	30.90 ± 1.21
E3	9.30 ± 0.37	17.78 ± 0.87	27.41 ± 1.03	32.77 ± 1.27
E4	12.38 ± 0.67	24.72 ± 1.18	32.70 ± 0.79	43.65 ± 1.98
E5	20.24 ± 1.44	40.60 ± 1.25	54.10 ± 1.57	68.92 ± 1.67
E6	15.20 ± 0.79	34.40 ± 0.85	48.52 ± 1.71	62.36 ± 1.68
E7	10.70 ± 0.47	20.10 ± 0.27	28.42 ± 0.80	36.80 ± 0.98
AscA	10.50 ± 0.72	20.70 ± 0.31	32.90 ± 0.18	46.60 ± 0.32
GA	21.80 ± 0.41	42.80 ± 1.88	58.61 ± 1.20	70.10 ± 1.46

Table 5: Reducing power of extract and subcomponents E1–E7 isolated from ET.

Component	AscAE (mg/g AscA)
CE	40.20 ± 1.10
PE	14.32 ± 0.42
ET	62.60 ± 1.55
BU	26.60 ± 0.51
WT	5.72 ± 0.26
E1	20.40 ± 0.77
E2	28.20 ± 1.22
E3	46.20 ± 1.18
E4	61.60 ± 1.11
E5	84.40 ± 2.10
E6	75.20 ± 1.50
E7	41.60 ± 0.95

The antioxidant activity of subcomponents E1–E7 isolated from ET in the β-carotene-linoleic acid system is shown in Table 4. At all experiment concentrations, E5 shows the strongest antioxidant activity followed by E6, E4, E7, E3, and E2 with E1 being the weakest. Among the seven subcomponents E5 and E6 show much stronger antioxidant activity than ET ($P < 0.05$), while the antioxidant activity of E5 is 68.92% at 200 μg/mL close to the synthesized antioxidant GA.

3.4.3. Reducing Power. Table 5 shows the reducing power of the extract from *S. clava* and subcomponents E1–E7 isolated from ET. The reducing powers of all the samples demonstrate a pattern similar to DPPH scavenging activity and antioxidant activity in the β-carotene-linoleic acid system. Among all the extracted components, ET is the highest in the reducing power ($P < 0.05$) and the reducing power of the seven subcomponents isolated from ET is in the following order: E5 > E6 > E4 > E3 > E7 > E2 > E1.

3.5. Structural Identification of Compound. Compound 1, colorless needle crystal, is identified as $C_{27}H_{46}O$ with EI-MS and NMR. δ3.47 in ^{1}H NMR, δ7.18 in ^{13}C NMR, and DEPT show that there is hydroxyl in the molecule. δ5.31 in ^{1}H NMR, δ140.18 and δ121.7 in ^{13}C NMR, and DEPT further confirm the double bond in the molecule. m/z 255 and 147 in MS combined with δ5.31 (1H, H-7), δ3.56 (1H, m, H-3), δ1.01 (3H, s, Me-19), and δ0.88 (3H, s, Me-18) in ^{1}HMMR, δ140.8 (s, C-8), δ121.7 (d, C-7), and δ71.8 (d, C-3) in ^{13}C NMR, and DEPT referred to the physical constant and spectral data reported by Tsuda and Schroepfer Jr [19]; compound 1 was identified as cholesteric-7-en-3β-ol, which was isolated from *S. clava* for the first time.

Compound 2, colorless needle crystal, mp 244~246°C is identified as $C_{27}H_{46}O_2$ with EI-MS and NMR. The two signals of methyl group connected with quaternary carbon (δ1.26, s; δ0.71, s) and three signals of methyl group connected with tertiary carbon (δ0.91, d; δ0.87, d; δ0.85, d) show that this compound is sterol. The signal of 19-Me at δ1.26 shifts to a lower field in contrast to cholesterol, which proves the existence of 6β-OH. Meanwhile, in contrast to cholesterol, signal (δ4.18) of 3α-H also shifts to a lower field and so is

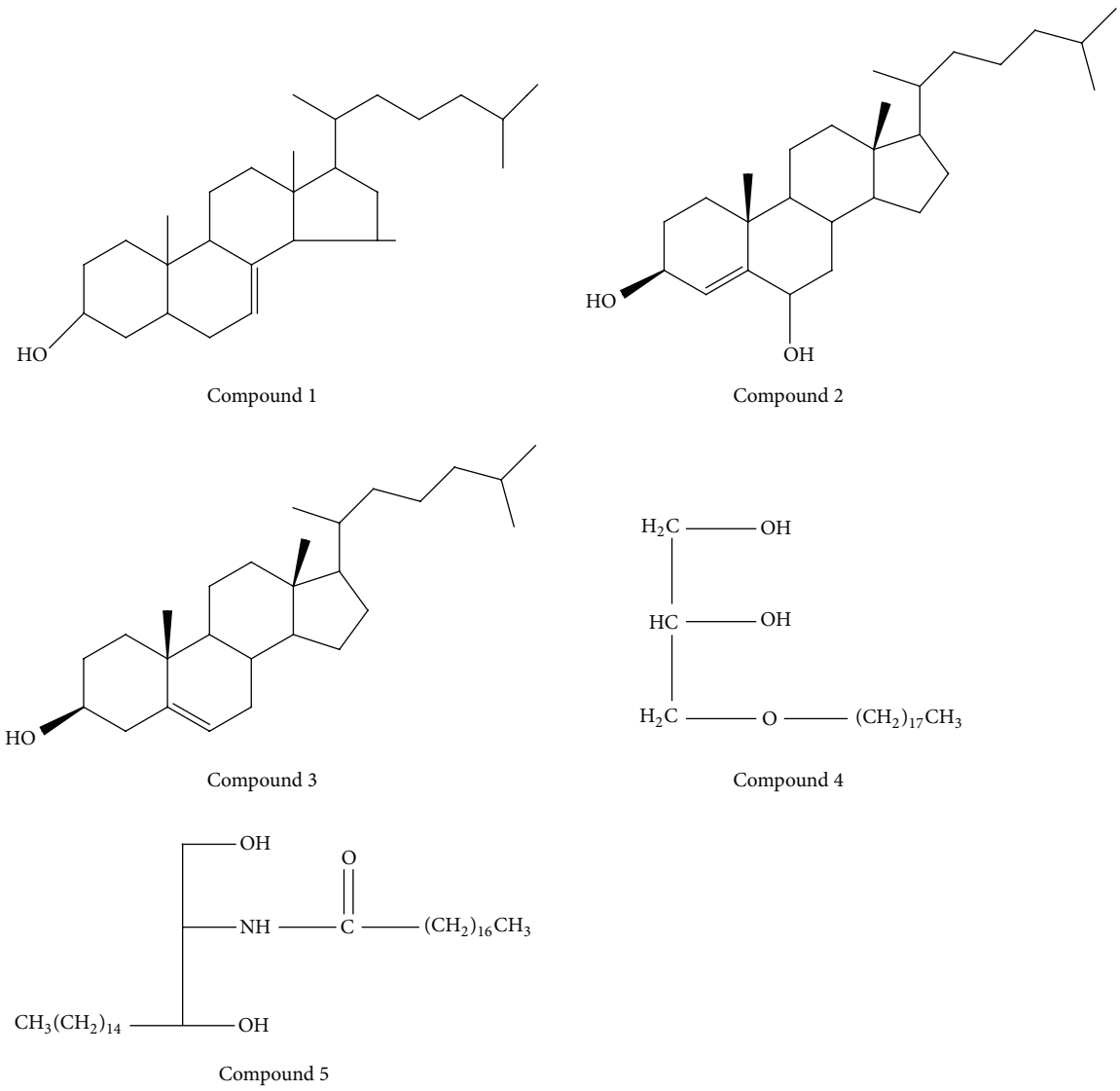

Figure 4: Structures of compounds.

proved of 4,5-double bond considering single peak δ5.55. Compound 2 is identified as cholesteric-4-en-3β, 6β-diol, referring to the physical constant and spectral data reported by Wahidullah et al. [20], which is isolated from *S. clava* for the first time.

Compound 3 is a white needle crystal, mp 148~149°C, purplish red at 10% sulfuric acid-ethanol solution. The multiple peaks at δ3.53 in ^{1}H NMR is the characteristic signal of 3α-H in sterol, while the multiple peaks at δ5.35 is signal of H-6. In addition, five methyl group signals appear in high field of ^{1}H NMR, among which the δ1.01, s and δ0.68, s belong to 19-Me and 18-Me, respectively, δ0.91, d is the signal of 21-Me, and δ0.84, d and δ0.82, d belong to 26-Me and 27-Me, respectively. Contrasting the physical constant and spectral data with Su et al. [21], compound 3 is identified as cholesterol.

Compound 4 is white powder, mp 68~69°C, purplish red at 10% sulfuric acid-ethanol solution. The molecular weight is 345 according to EI-MS. As shown in ^{13}C NMR spectrum, δ64.1 (t, C-1), δ31.9 (t, C2), δ29.6 (t, C3-C15), δ26.0 (t, C16), δ22.6 (t, C17), and δ14.1 (t, C18) are characteristic signals of long chain fatty acid, and δ72.2 (C-1), δ70.6 (C-2), and δ71.8 (C-3) belong to glycerol; so this compound should be a long chain triglyceride. However, there is no signal of ester carbonyl; therefore, this compound is identified as glycidyl ether, agreeing with Yang et al. [22] and Wang et al. [23], and is confirmed as batilol, which is isolated from *S. clava* for the first time.

Compound 5, molecular formula is $C_{36}H_{71}NO_3$ according to EI-MS and NMR. δ6.37 (1H) in ^{1}H NMR spectrum and δ173.9 (C=O) in ^{13}C NMR spectrum proves the existence of acylamino, and δ54.5 in ^{13}C NMR spectrum indicates that what is connected with nitrogen is methyne. δ2.02 (2H) in ^{1}H NMR indicates that there are two –OH in the compound, and δ74.74 and δ62.40 in ^{13}C NMR indicate that –CH– and –CH_2– connect with –OH, respectively. Meantime, a strong

hydrogen signal of δ1.26 in ^{1}H NMR spectrum and two signals $-CH_3-$ of δ0.84 (6H) determine two long chain alkyl groups. This compound is identified as ceramide referring to the physical constant and spectral data reported by Yu and Yang [24].

The structures of these compounds are shown in Figure 4. As reported by Cai et al. [25], the number and position of hydroxyl groups largely determined radical scavenging activity of phenolic compounds. Compounds 1, 2, and 3 all have only one phenolic hydroxyl group, and there is no functional groups in orthoposition; little steric hindrance effect increased their contact with free radicals.

4. Conclusion

The immunomodulatory and antioxidant activity of crude and fractionated extracts of the ascidian *Styela clava* were determined by *in vitro* screening. All these extracts demonstrate immunomodulatory activity through increasing the proliferation rate of spleen lymphocyte and macrophages, as well as the NO release activities of macrophages. Among them the petroleum ether fraction shows the strongest immune active *in vitro*. The ethyl acetate fraction (ET) was much higher in its antioxidant activity in DPPH system, reducing power assay and β-carotene-linoleic acid system compared with the other fractions, and its subcomponent E5 demonstrated the strongest antioxidant activity as well as reducing power, higher than the positive control AscA and close to the synthesized GA. ET was isolated systemically and five compounds were separated as (1) cholesteric-7-en-3β-ol, (2) cholesteric-4-en-3β,6β-diol, (3) cholesterol, (4) batilol, and (5) ceramide, among which (1), (2), and (4) were isolated for the first time from *S. clava*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ju Bao and Chen Bin contributed equally to this project.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (no. 31070368) and the Science and Technology Department of Shandong Province (BS2010HZ022 and ZR2011DL008).

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