

Glycolipids from marine macroalgae: extraction methodology, isolation and biological activity

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Abstract

Considering the yield, concentration of glycolipids, moisture absorption and moisturizing activity of the extract, *Bangia fusco-purpurea* was selected from 8 species of marine macroalgae (*Bangia fusco-purpurea*, *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp.) as the raw material for the extraction methodology, isolation and purification of glycolipids. Through single factor and response surface experiments, the suitable extraction conditions (the solid-to-liquid ratio, extraction temperature, extraction time and ultrasonic power) of the glycolipids from *Bangia fusco-purpurea* was: 1:27 g/mL, 49 °C, 98 min and 500 W. Using the optimized process, the yield of extract and concentration of glycolipids in extract obtained were 28.1% and 116.9 µg/mL. Further, three compounds (H₁₂, H₂₂ and H₃₃) were obtained from *Bangia fusco-purpurea* by liquid-liquid extraction, silica gel column chromatography and preparation thin layer chromatography, namely hexadecan-1-yl-1-O- α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)- α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)- α -D-arabinopyranoside, β -Gal-(1-3)- β -Xyl, and docosan-1-yl-1-O- α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)-3-O-acetyl- α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)- α -D-arabinopyranoside. This is the first report that these three compounds were isolated from *Bangia fusco-purpurea*. And those two with arabinopyranosyloxy (H₁₂ and H₃₃) were obtained from marine macroalgae for the first time. Also, the glycolipids from *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp. were preliminarily determined by TLC and HPLC, and compared with glycolipid standard (MGDG, SQDG, DGDG), and found that MGDG or SQDG was existed in 7 species of marine macroalgae.

1 Introduction

Glycolipids are a class of compounds formed by one or more monosaccharide residues linked to lipid moieties, monoacyl or diallylglycerols by glycosidic bonds (Yang, 1998). They widely exist in various organisms. Recently, with the prominence of ecological or pharmaceutical interest activities, the interest in glycolipids of marine origin such as marine macro-micro algae and microorganism has grown rapidly (Hammami et al., 2010; Li et al., 2016; Plouguerné et al., 2014).

In fact, researchers have been interested in the glycolipids from marine macroalgae because of their multiple activities for a long time (Jamieson and Reid, 1976). It has been reported that the sugar was mainly galactose (Jin et al., 2011; Kim et al., 2007; Lopes et al., 2019; Mattos et al., 2011; Tang et al., 2002), followed by sulfo-rhamnose (Al Fadhli et al., 2006; Arunkumar et al., 2005; Cui et al., 2001; Lopes et al., 2019; Mattos et al., 2011), and glucose existed only in very few glycolipids from some marine macroalgae (Wu et al., 2009), for example, *Ascophyllum nodosum* (Jamieson and Reid, 1976), *Grateloupia turuturu* (Da Costa et al., 2021), *Sargassum carpophyllum* (Tang et al., 2002), *Sargassum thunbergii* (Jin et al., 2011; Kim et al., 2007), *Caulerpa racemosa* (Mattos et al., 2011), *Chondria armata* (Al Fadhli et al., 2006), *Palmaria palmata* (Lopes et al., 2019), *Sargassum hemiphylum* (Cui et al., 2001), *Sargassum wightii* (Arunkumar et al., 2005), *Sargassum fulvellum* (Wu et al., 2009), etc. Up to now, arabinose was not found in the glycolipids from seaweeds.

In our previous research (Sun et al., 2021), glycosylglycerolipids with antifungal activity were screened from *Codium fragile*, *Hizikia fusiforme*, *Laminaria japonica*, *Pelvetia siliquosa*, *Porphyra haitanensis* and *Undaria pinnatifida*. Also, the sugar was galactose in the two glycosylglycerolipids isolated from *Pelvetia siliquosa* and *Porphyra haitanensis*. In the present study, *Bangia fusco-purpurea*, *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp. were selected as raw material for extracting glycolipids. There was no report on their glycolipids before, and this work hopes to obtain new glycolipids from these marine macroalgae.

2.1 Marine macroalgae

Bangia fusco-purpurea (Rhodophyta), *Gelidium amansii* (Rhodophyta), *Gloiopeltis furcata* (Rhodophyta), *Gracilaria lemaneiformis* (Rhodophyta), *Gracilaria* sp. (Rhodophyta), *Palmaria palmata* (Rhodophyta), *Porphyra yezoensis* (Rhodophyta) and *Scagassum* sp. (Phaeophyta) were purchased from a wholesaler. They were quickly rinsed with distilled water, blotted on tissue papers, dried in a blast drying oven. Dried macroalgae materials were cut into small pieces (ca. 2.0 cm of length) or crumbled. And then these pieces were ground to make powder using a blender for 1 min. The powder conservation was at -20°C until the extraction.

2.2 Chemical Reagents

monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) standards were obtained from Avant Polar Lipids (USA). The other solvents or compounds were analytically pure, and from Sinopharm Chemical Reagent Co., Ltd..

2.3 Extraction

2.3.1 Single factor experiments

10 g powder of macroalgae was poured into a certain volume of methanol solution (volume fraction), and placed on an ultrasonic cleaner for ultrasonic extraction for a certain time under different ultrasonic power and temperature. After filtration, repeat the above operation twice for the filter residue. The filter solution was combined and evaporated to dryness under reduced pressure. In this way, the extracts of marine macroalgae were obtained. The extraction process of the extracts containing glycolipids was optimized. In this process, the solid-to-liquid ratio, extraction temperature, time, ultrasonic power and volume fraction of methanol were set according to Table 1. When conducting the solid-liquid ratio experiments, extraction temperature, time, ultrasonic power and volume fraction of methanol were set to 45 °C, 120 min, 500 W and 85%. In the extraction temperature experiments, the solid-liquid ratio with higher yield of extract and the content of glycolipids in the solid-to-liquid ratio experiments was selected as the value of the solid-liquid ratio, while extraction time, ultrasonic power and volume fraction of methanol were set to 120 min, 500 W and 85%. For the extraction time experiments, ultrasonic power and volume fraction of methanol were set to 500 W and 85%, but the solid-liquid ratio and extraction temperature were set according the results of the solid-to-liquid ratio experiments and the extraction

temperature experiments, namely, the solid-liquid ratio and extraction temperature with higher yield and the content of glycolipids in the solid-liquid ratio experiments and the extraction temperature experiments were selected. Other factor experiments were performed according to similar methods. Glycolipids in these extracts were determined by silica gel thin layer chromatography. 0.1 g extract was dissolved in 20 mL of methanol and diluted 10 times to obtain glycolipids solution. 1 mL of 5% phenol and 5 mL of concentrated sulfuric acid were added to 2 mL glycolipids solution successively, and then take a water bath at 80 °C for 30 minutes. After cooling to room temperature, the absorbance was measured at 480 nm (or 490 nm). According to the standard curve, the concentration of glycolipids in extract was calculated.

Table 1
Factors and levels of single factor experiments

Factor Level	Solid-to-liquid ratio (g/mL)	Temperature (°C)	Time (min)	Ultrasonic power (W)	Volume fraction of methanol (%)
1	1:10	25	30	250	45
2	1:15	35	60	300	55
3	1:20	45	90	350	65
4	1:25	55	120	400	75
5	1:30	65	150	450	85
6	1:35	75	180	500	95

2.3.2 Response Surface Methodology

The optimum extraction process for glycolipids from *Bangia fusco-purpurea* was evaluated by Response Surface Methodology, including solid-to-liquid ratio, temperature, time and ultrasonic power. The three levels for each factor showed in Table 2.

Table 2
Factors and levels of Response Surface Methodology

Factor Level	Solid-to-liquid ratio (g/mL)	Temperature (°C)	Time (min)	Ultrasonic power (W)
	A	B	C	D
1	1:20	35	60	400
2	1:25	45	90	450
6	1:30	55	120	500

2.3.3 Different solvent

According to the results of the response surface optimization experiments mentioned-above, solid-to-liquid ratio, temperature, time and ultrasonic power were set.

2.4 isolation and purification

4.0 g of glycolipids extracts were dissolved in 400 mL of buffer solution with pH 2 (The preparation method of buffer solution was as follows: 3.7275 g KCl and 25.00 g NaCl were dissolved in 200 mL of distilled water, and poured into a 1000 mL of volumetric flask. And then 6.5 mL of 2 mol/L hydrochloric acid solution was added, adjust pH to 2 and fix the volume to 1000 mL.), and poured into the separating funnel. Then 200 mL of hexane was added and vibrated fully, to stand for 2–4 h. The upper phase (hexane phase, HE) was collected, and the remaining phase was extracted again with hexane. After repeated extraction with hexane for 3 times, dichloromethane was added and extracted for times. After the dichloromethane extraction was completed, ethyl acetate was used for extraction according to the above method. Finally, n-butanol was added for extraction. According to this liquid-liquid extraction method, hexane phase (HE), dichloromethane phase (DE), ethyl acetate phase (EE), and n-butanol phase (BE) were obtained in turn. These phases were combined according to the profiles of MGDG, DGDG and SQDG standards in silica gel TLC, and target portions were determined.

According to the behavior of TLC, target portions containing glycolipids were isolated by silica gel column chromatography (100–200 mesh, 4.0 × 40 cm), respectively. First, it was eluted with petroleum ether, petroleum ether: ethyl acetate (2:8, v:v) and dichloromethane twice the column volume in order to remove impurities after the portion was loaded on the top of the silica gel column. Subsequently, dichloromethane: methanol (2:3, v:v) was used as eluent for elution, and 20 mL of eluents was collected in each tube. The elution volume was 2.5 times of the column volume and the flow rate was 1 mL/min. Subsequently, all eluents were detected and combined using silica gel TLC to obtain components contained glycolipids. These target components were further purified by preparation TLC. Finally, these prepared samples were compared with R_f of TLC and the residence time of HPLC of the standard, and identified by nuclear magnetic resonance spectroscopy.

2.5 Silica gel thin layer chromatography

The standards (MGDG, DGDG, SQDG), the extracts of macroalgae, portions, eluents and prepared samples were respectively spotted on the chromatographic plate, developed with chloroform: methanol: water (65:15:3, v:v:v), sprayed phenol-sulfuric acid for color development or developed with iodine vapor, and dried at 110 °C for 20 minutes.

2.6 Moisture-absorption and moisture-retention activity assay

2.6.1 Moisture-absorption measurement

0.5 g of sodium alginate, sorbierite, glycerin and macroalgae extract, which previously dried to constant weight, were accurately weighed, respectively, and put in Petri dishes with a diameter of 5 cm. And then,

these Petri dishes were placed in the dryer. There was saturated sodium carbonate solution in the dryer (relative humidity (RH) is 43%). The placement time was set to 2 h, 4 h, 8 h, 12 h, 24 h and 36 h, and the moisture absorption rate was calculated according to the mass difference of samples before and after placement. According to the same method-above, the moisture absorption test of trehalose, sodium alginate, sorbitol, glycerol, and macroalgae extracts at a RH of 81% (saturated ammonium sulfate solution in the dryer) was carried out.

Moisture-absorption rate = $[(m_n - m_0)] \times 100\%$, where m_0 and m_n were the mass of test sample before and after placement respectively.

2.6.2 Moisture-retention measurement

First, tested sample (trehalose, sodium alginate, sorbitol, glycerol, and macroalgae extract) was prepared with a mass concentration of 20%. Then, 0.6 g of tested sample solutions were accurately weighed and put into weighing bottles respectively. These weighing bottles were placed in a dryer with dry silica gel for 2 h, 4 h, 8 h, 12 h, 24 h and 36 h, and the moisture-retention rate was calculated according to the mass difference of samples before and after placement.

Moisture-retention rate = $(H_n/H_0) \times 100\%$, where H_0 and H_n were the mass of test sample before and after placement respectively.

2.7 Data process and statistical analysis

Cells numbers of microalgae were counted by hemocytometer. All the data of the growth assays in this study were analyzed by ANOVA and Tukey's test.

2.1 Marine Macroalgae

Bangia fusco-purpurea (Rhodophyta), *Gelidium amansii* (Rhodophyta), *Gloiopeltis furcata* (Rhodophyta), *Gracilaria lemaneiformis* (Rhodophyta), *Gracilaria* sp. (Rhodophyta), *Palmaria palmata* (Rhodophyta), *Porphyra yezoensis* (Rhodophyta) and *Scagassum* sp. (Phaeophyta) were purchased from a wholesaler. They were quickly rinsed with distilled water, blotted on tissue papers, dried in a blast drying oven. Dried macroalgae materials were cut into small pieces (ca. 2.0 cm of length) or crumbled. And then these pieces were ground to make powder using a blender for 1 min. The powder conservation was at -20°C until the extraction.

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Table 2. Factors and levels of Response Surface Methodology

Factor Level	Solid-to-liquid ratio (g/mL) A	Temperature (°C) B	Time (min) C	Ultrasonic power (W) D
1	1:20	35	60	400
2	1:25	45	90	450
6	1:30	55	120	500

2.3.3 Different solvent

According to the results of the response surface optimization experiments mentioned-above, solid-to-liquid ratio, temperature, time and ultrasonic power were set.

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2.6.1 Moisture-absorption measurement

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Moisture-absorption rate = $[(m_n - m_0)] \times 100\%$, where m_0 and m_n were the mass of test sample before and after placement respectively.

2.6.2 Moisture-retention measurement

First, tested sample (trehalose, sodium alginate, sorbitol, glycerol, and macroalgae extract) was prepared with a mass concentration of 20%. Then, 0.6 g of tested sample solutions were accurately weighed and put into weighing bottles respectively. These weighing bottles were placed in a dryer with dry silica gel for 2 h, 4 h, 8 h, 12 h, 24 h and 36 h, and the moisture-retention rate was calculated according to the mass difference of samples before and after placement.

Moisture-retention rate = $(H_n / H_0) \times 100\%$, where H_0 and H_n were the mass of test sample before and after placement respectively.

2.7 Data process and statistical analysis

Cells numbers of microalgae were counted by hemocytometer. All the data of the growth assays in this study were analyzed by ANOVA and Tukey's test.

3 Results

3.1 Single factor experiments

Solid-to-liquid ratio, temperature, time, ultrasonic power and volume fraction of methanol have effects on the yield of extract and the concentration of glycolipids in extract from *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp., and *Porphyra yezoensis* (Fig. 1). Among them, solid-to-liquid ratio has the most significant ($p < 0.05$) effect on the yield of extract and the concentration of glycolipids in extract from 4 species of marine macroalgae, while the significance of the other three factors varies with the species of marine macroalgae. Within limits, the yield of extract from 4 species of marine macroalgae increased with the increase of factor level. As the factor level continues to increase, the yield of extract showed a downward trend. The change trend of the concentration of glycolipids in extract with the increase of factor level was similar to that of the yield of extract from 4 species of marine macroalgae, however, it was not exactly the same. In this study, the maximum yield of extract and the maximum concentration of glycolipids in extract from 4 species of marine macroalgae were usually achieved at different factor levels. For example, the extraction time was respectively 180 min and 60 min, 60 min and 120 min, 120 min and 180 min, 60 min and 90 min when the yield of extract and concentration of glycolipids in extract from *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* reach the maximum. This showed that the types of glycolipids in the extract obtained at different factor levels were likely to be different. Therefore, in the extraction experiments, the yield of extract and the concentration of glycolipids in extract should be paid attention. Comprehensively consider them to select appropriate factor levels in the extraction of glycolipids from marine macroalgae. The specific level of the factor when the yield of extract and the concentration of glycolipids in the extract reach the maximum showed in Table 3.

Table 3 The factor level at the maximum yield of extract and the concentration of glycolipids in extract in single

Marine macroalgae	<i>Bangia fusco-purpurea</i>		<i>Gracilaria lemaneiformis</i>	
	Yield	Concentration of glycolipids	Yield	Concentration of glycolipids
Solid-to-liquid ratio	1:25 g/mL	1:25 g/mL	1:35 g/mL	1:25 g/mL
Temperature	45°C	45°C	45°C	75°C
Time	180 min	60 min	60 min	120 min
Ultrasonic power	400 W	450 W	350 W	450 W
Volume fraction of methanol	65%	85%	45%	85%
Marine macroalgae	<i>Gracilaria</i> sp.		<i>Porphyra yezoensis</i>	
	Yield	Concentration of glycolipids	Yield	Concentration of glycolipids
Solid-to-liquid ratio	1:30 g/mL	1:30 g/mL	1:30 g/mL	1:10 g/mL
Temperature	75°C	55°C	55°C	75°C
Time	120 min	180 min	60 min	90 min
Ultrasonic power	350 W	350 W	500 W	500 W
Volume fraction of methanol	85%	55%	55%	95%

It can be clearly seen that *Bangia fusco-purpurea* and *Gracilaria* sp. have higher yield of extract (20%–36%, the yield was 17.1% except when the solid-to-liquid ratio was 1:10) and the concentration of glycolipids among 4 species of marine macroalgae. And in extraction process of glycolipids from these two species of marine macroalgae, solid-to-liquid ratio, temperature, time and ultrasonic power were important factors. It is particularly emphasized here that there were differences in of glycolipids in the extract obtained with different volume fraction of methanol. At the same time, it was also found that the yield of extract and the concentration of glycolipids in extract from *Bangia fusco-purpurea* and *Gracilaria* sp. at 85% of volume fraction of methanol were higher than those at other volume fraction of methanol. Therefore, in the optimization experiments by response surface methodology, the effects of solid-to-liquid ratio, temperature, time and ultrasonic power were further considered on the yield of extract and the concentration of glycolipids in extract from *Bangia fusco-purpurea*.

Furthermore, the effects of different solvents on the yield of extract from *Bangia fusco-purpurea* and *Gracilaria* sp. were also analyzed. It could be clearly seen from Fig. 2 that the higher yields could be obtained by using 95% ethanol, 95% methanol and 85% methanol as the extraction solvent, especially the highest yield of extract was obtained when 95% methanol was used as the extraction solvent. Therefore, 95% methanol was used as the extraction solvent to obtain glycolipids extract from *Bangia fusco-purpurea* and *Gracilaria* sp. in follow-up experiments.

In addition, from the data of single factor experiments mentioned-above, solid-to-liquid ratio, temperature, time, ultrasonic power and volume fraction of methanol were set to 1:30, 45°C, 120 min, 500 W and 85%, respectively, for the extraction of glycolipids in *Gelidium amansii*, *Gloiopeltis furcata*, *Palmaria palmata*, and *Scagassum* sp.. Also, it was found that these four species of marine macroalgae contained glycolipids. The yields of extracts from those macroalgae were between 2.4% and 8.5%, and were significantly lower than that from 4 species of marine macroalgae such as *Bangia fusco-purpurea*.

3.2 Properties of moisture absorption and moisture retaining

The moisture absorption and moisturizing rate of extract from *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* were carried out. In Fig. 3, under RH 43% and 81%, the moisture adsorption rate of extracts from 4 species of marine macroalgae was close to that of sorbierite and sodium alginate, and was lower than that of glycerin, especially their moisturizing rate was significantly ($p < 0.05$) lower than that of glycerol after 54 h. Of note, under 43%, the moisture adsorption rate of extracts from 4 species of marine macroalgae was significantly higher than that of extract from *Laminaria japonica* (Zhao et al., 2012). Among these extracts from 4 species of marine macroalgae, the moisture absorption rate of extract from *Gracilaria lemaneiformis* was higher than that of other three marine macroalgae at RH 43%; the moisture absorption rate of extract from *Bangia fusco-purpurea* was much larger than other three marine macroalgae at RH81%.

In the first 42 hours, the moisturizing rate of extract from *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* was very close to that of glycerol, glycerin and

sodium alginate. After 54 h, their moisturizing rate was markedly lower than that of glycerol, while was still close to that of sodium alginate.

To sum up, the glycolipids content and moisture absorption activity of *Bangia fusco-purpurea* were superior to the other three species of marine macroalgae. Therefore, the extraction process of *Bangia fusco-purpurea* glycolipids was further optimized in the subsequent experiments.

3.3 Response surface experiments

The linear parameters A (solid-to-liquid ratio), B (extraction temperature), C (extraction time), D (ultrasonic power), quadratic parameters (A^2 , B^2 and C^2), the interaction between solid-to-liquid ratio and extraction time (AC), the interaction between solid-to-liquid ratio and ultrasonic power (AD), the interaction between extraction temperature and ultrasonic power (BD), and the interaction between extraction time and ultrasonic power (CD) significantly affected the yield of extract from *Bangia fusco-purpurea* ($P < 0.05$). The equation obtained was given in Eq. 1.

$$Y_{\text{yield}} = 27.9 + 0.3133A + 0.5000B + 0.2075C + 0.1775D + 0.0400AB + 0.3175AC - 0.2675AD + 0.0475BC + 0.3425BD - 0.2925CD - 0.7737A^2 - 0.7212B^2 - 0.6150C^2 - 0.575D^2 \quad (1)$$

In Fig. 4a, the yield of extract from *Bangia fusco-purpurea* increased as solid-to-liquid ratio and extraction temperature increased to a certain point. Similarly, the yield of extract increased to a certain point with the increase of extraction time and ultrasonic power. The interaction of solid-to-liquid ratio and extraction time (AC), and the interaction between extraction time and ultrasonic power (CD) led to synergistic effect that was more significant in enhancing yield. It showed the coefficient for extraction time and ultrasonic power (or the coefficient for and solid-to-liquid ratio and extraction time) was higher at 0.3425 (or 0.3175, in Eq. 1) than for other interactions. According to Eq. 1, the optimum yield of 28.02% was predicted at the solid-to-liquid ratio of 1:27.59 g/mL, extraction temperature of 49.39 °C, extraction time of 88.36 min, and ultrasonic power of 500 W. The verification findings showed the yield of 28.09%.

The linear parameters A (solid-to-liquid ratio), C (extraction time), quadratic parameters (A^2 , B^2 , C^2 and D^2), the interaction between solid-to-liquid ratio and extraction temperature (AB), the interaction between solid-to-liquid ratio and extraction time (AC), and the interaction between extraction temperature and ultrasonic power (BD) significantly affected the concentration of glycolipids in extract from *Bangia fusco-purpurea* ($P < 0.05$). The equation obtained was given in Eq. 2.

$$Y_{\text{yield}} = 120.71 - 5.68A - 0.3169B + 5.30C - 0.2942D + 1.42AB - 3.76AC - 0.3325AD + 0.6525BC - 1.67BD - 0.7250CD - 12.32A^2 - 8.36B^2 - 7.09C^2 - 3.64D^2 \quad (2)$$

The concentration of glycolipids in extract from *Bangia fusco-purpurea* increased as solid-to-liquid ratio and extraction time increased to a certain point. Also, they increased to a certain value with the increase of extraction temperature and ultrasonic power. The interaction between solid-to-liquid ratio and extraction temperature (AB), the interaction between solid-to-liquid ratio and extraction time (AC), and the

interaction between extraction temperature and ultrasonic power (BD) significantly affected the increasing of the concentration of glycolipids in extract. And it can be clearly seen that the coefficient for solid-to-liquid ratio and extraction temperature was higher at 1.42 (in Eq. 2) than that for other interactions. According to Eq. 2, the optimum concentration of glycolipids of 116.895 µg/mL was predicted at the solid-to-liquid ratio of 1:27.035 g/mL, extraction temperature of 45.36 °C, extraction time of 98.17 min, and ultrasonic power of 445 W. The verification findings showed the concentration of glycolipids of 117.043 µg/mL.

The results of validation experiments showed that the verification values of the yield and the concentration of glycolipids in the extract from *Bangia fusco-purpurea* were closed to the prediction of those. If the prediction error value was less than 5%, the verification results were acceptable and verified. All things considered, the solid-to-liquid ratio, extraction temperature, extraction time and ultrasonic power was set as 1:27 g/mL, 49 °C, 98 min and 500 W, respectively. By this process, the yield of extract and the concentration of glycolipids were 28.1% and 116.9 µg/mL.

3.4 Isolation and purification

In order to obtain glycolipids from *Bangia fusco-purpurea*, the extract was portioned by liquid-liquid extraction. Results showed that all portions (namely was recorded as hexane phase, dichloromethane phase, ethyl acetate phase and n-butanol phase) which isolated from extract from *Bangia fusco-purpurea* contained glycolipids. The total yields of these portions isolated from extract from *Bangia fusco-purpurea* were 55.35%.

And four portions isolated from extract from *Bangia fusco-purpurea* were investigated by TLC and HPLC (supplementary materials) (Table 4 and Fig. 5), and compared with standard MGDG, SQDG and DGDG. The behavior of TLC was different from that of the standards MGDG and DGDG, and close to that of standard SQDG. It showed that these portions were likely to contain compounds different from the standards. In HPLC (see supplementary materials), the chromatographic peak of these portions exhibited different retention time from those of MGDG and DGDG, and close to that of standard SQDG reported by Honda et al. (2016), which indicates that there should contain sulfonoglycolipid and may be new types of glycolipids in *Bangia fusco-purpurea*. In reference (Honda et al., 2016), the retention time of MGDG, SQDG and DGDG was 7.5–8.2 min, 10.1–11.0 min, and 14.1–14.6 min, respectively.

Table 4 The percentage (%) and glycolipids determination of portions from the extract from marine macroalgae by the liquid-liquid extraction

Percentage Glycolipids	Portions			
	Hexane phase	Dichloromethane phase	Ethyl acetate phase	N-butanol phase
<i>Bangia fusco-purpurea</i>	8.64 + Unknown, SQDG	13.5 + Unknown, SQDG	6.71 + Unknown, SQDG	26.5 + Unknown, SQDG
<i>Gracilaria lemaneiformis</i>	0.73 + SQDG	1.52 + SQDG	0.93 + SQDG	10.3 -
<i>Gracilaria</i> sp.	53.62 + SQDG	4.67 + SQDG	1.53 + SQDG	4.2 + SQDG
<i>Porphyra yezoensis</i>	4.50 + MGDG, SQDG	12.7 + MGDG, SQDG	2.91 + SQDG	34.70 -
<i>Gelidium amansii</i>	-	65.25 -	8.69 + MGDG	4.87 + MGDG, SQDG
<i>Gloiopeltis furcata</i>	-	23.7 + SQDG	1.06 + SQDG	20.1 -
<i>Palmaria palmata</i>	-	22.7 + MGDG	41.9 + MGDG, SQDG	24.4 + SQDG
<i>Scagassum</i> sp.	-	64.2 + MGDG, SQDG	8.13 -	5.95 + SQDG

Note: “+” indicates positive, namely contain glycolipids; “-” indicates negative, namely do not contain glycolipids. MGDG, DGDG and SQDG were identified by HPLC (supplementary materials).

Subsequently, these portions obtained from the extract from *Bangia fusco-purpurea* were subsequently isolated by the silica gel column chromatograph (2.0 × 40 cm, 100–200 mesh) with dichloromethane/methanol (2:3, v.v) as eluent. The collected eluents were detected by silica gel thin layer chromatography. In Fig. 5, for dichloromethane phase, the tube 8 - tube 14 (H₁) was collected; for ethyl acetate phase, 2 - 8 tubes (H₂) were target eluents; for n-butanol phase, tube 9 - tube 13 (H₃) were collected (Fig. 6). Furthermore, H₁, H₂ and H₃ were isolated by preparation thin-layer chromatography. In Fig. 7, it could be clearly seen that the strips of H₁, H₂ and H₃ were not in the same positions as that of the three standards MGDG, DGDG and SQDG in PTLC. Finally, H₁₂ (20.1 mg), H₂₂ (25.6 mg) and H₃₃ (33.6 mg) were prepared.

In addition, the extracts of *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp. were also isolated by liquid-liquid extraction. And all portions isolated from those extracts were determined by TLC and/or HPLC (supplementary materials) and compared with standard MGDG, SQDG and DGDG (Table 4 and Fig. 5). Among them, SQDG was common in *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp.. This is the first time to report the kinds of glycolipid from 7 marine macroalgae.

3.5 Identification

Result suggested that H₁₂, H₂₂ and H₃₃ should be other glycolipids or compound different from three standards (Fig. 7). The molecular structure of H₁₂, H₂₂ and H₃₃ were identified by nuclear magnetic resonance spectroscopy and NMR.

H₁₂, white solid, C₃₁H₅₈O₁₃, ESI-MS m/z: 661.37 [M + Na]⁺. ¹H-NMR and ¹³C-NMR data were listed in Table 5. These spectral data were consistent with the reported results (Rajesh and Vijai, 2006), H₁₂ was identified as hexadecanyl-1-*O*-α-D-arabinopyranosyloxy (1→4)-α-D-arabinopyranosyloxy (1→4)-α-D-arabinopyranoside.

Table 5a NMR spectroscopic data of H₁₂ and H₃₃ in CDCl₃

H ₁₂			H ₃₃		
C. No.	H		C. No.	H	
	δ ¹³ C NMR	δ ¹ H NMR	δ ¹³ C NMR	δ ¹³ C NMR	δ ¹ H NMR
C-1	68.6 (CH ₂)		C-1	70.6 (CH ₂)	
C-2	31.7 (CH ₂)	3.50-4.44 (17H, m, oxygenated-H)	C-2	33.6 (CH ₂)	3.49-4.31 (17H, m, oxygenated-H)
C-3 C-15	21.7-30.0 (CH ₂)		C-3-C21	22.4-30.0 (CH ₂)	
C-16	13.1 (CH ₃)	0.90 (3H, t, <i>J</i> = 6.8 Hz)	C-22	13.1(CH ₃)	0.91 (3H, t, <i>J</i> = 6.8 Hz)
Arabinose-1			Arabinose-1		
1'	104.2		1'	105.0	
2'	70.8		2'	70.8	
3'	70.1	5.03-5.10 (3H, d, <i>J</i> = 3.0 Hz, Ara-1*3)	3'	70.1	54.95-5.10 (3H, d, <i>J</i> = 3.0 Hz, Ara-1*3)
4'	75.8		4'	73.1	
5'	60.6		5'	60.6	
Arabinose-2			Arabinose-2		
1''	98.9		1''	99.1	
2''	71.1		2''	71.1	
3''	70.1	5.03-5.10 (3H, d, <i>J</i> = 3.0 Hz, Ara-1*3)	3''	70.1	4.95-5.10 (3H, d, <i>J</i> = 3.0 Hz, Ara-1*3)
4''	75.8		4''	71.2	
5''	61.2		5''	61.2	
			3''-OAc	22.4	
				173.2	
Arabinose-3			Arabinose-3		
1'''	98.5		1'''	98.1	
2'''	70.6		2'''	70.6	
3'''	70.8	5.03-5.10 (3H, d, <i>J</i> = 3.0 Hz, Ara-1*3)	3'''	70.8	4.95-5.10 (3H, d, <i>J</i> = 3.0 Hz, Ara-1*3)
4'''	70.2		4'''	70.2	
5'''	64.8		5'''	64.8	

Table 5b NMR spectroscopic data of H₂₂ in CDCl₃

C. No.	C	H. No.	H
	$\delta^{13}\text{C}$ NMR		$\delta^1\text{H}$ NMR
Galactose			
1	99.3	Gal-1	4.81 (1H, d, $J = 7.8$ Hz)
2	79.9		
3	71.5		
4			
5	68.6		
6	61.4		
Xylose			
1'	98.4	Xyl-1	4.62 (1H, d, $J = 7.8$ Hz)
2'	75.3		
3'	70.5		
4'	69.1		
5'	62.0		
			3.45-3.90 (11H, m, oxygenated-H)

H₂₂, white solid, C₁₁H₂₀O₁₀, ESI-MS m/z : 355.09 [M + Na]⁺. ¹H-NMR and ¹³C-NMR data of H₂₂ were consistent with that of β -Gal-(1→3)- β -Xyl (Trincone et al., 2003).

H₃₃, C₃₇H₇₀O₁₃, ESI-MS m/z : 745.47 [M + Na]⁺. Its ¹H-NMR and ¹³C-NMR data were the same as those reported in a literature (Rajesh and Vijai, 2006), so it was identified as docosanyl-1-*O*- α -D-arabinopyranosyloxy-(1→4)-3-*O*-acetyl - α -D-arabinopyranosyloxy-(1→4)- α -D-arabinopyranoside.

This was the first report of hexadecanyl-1-*O*- α -D-arabinopyranosyloxy (1→4)- α -D-arabinopyranosyloxy (1→4)- α -D-arabinopyranoside, β -Gal-(1→3)- β -Xyl and docosanyl-1-*O*- α -D-arabinopyranosyloxy-(1→4)-3-*O*-acetyl - α -D-arabinopyranosyloxy-(1→4)- α -D-arabinopyranoside isolated from *Bangia fusco-purpurea* (Fig. 8). Among them, these two glycolipids contained arabinose, and this is the first time to separate this type of glycolipid from marine macroalgae.

Due to the low preparation quality of H₁₂ and H₂₂, only H₃₃ was analyzed for moisturizing activity (Fig. 3). Results showed that the moisturizing rate of H₃₃ was very close to that of glycerol, glycerin and sodium alginate in the first 42 hours, although the moisture retaining properties of H₃₃ cannot last for a long time. During the whole experimental period, the moisturizing activity of H₃₃ was very close to that of *Bangia fusco-purpurea* extract, which indicated that this component was the material basis of *Bangia fusco-purpurea* extract with moisturizing activity.

4 Discussions

Glycolipids are compounds in which sugars are linked to glycerides by glycosidic bonds, which with different structures, such as monogalactosyl diacylcerol (MGDG) (Sun et al., 2021), monogalactosyl monoacylglycerol (MGMG) (Sanina et al., 2012), digalactosyl diacylglycerol (DGDG) (Plouguerné et al.,

2020), digalactosyl monoacylglycerol (DGMG) (Sun et al., 2021; Xu et al., 1992), sulfoquinovosyl diacylglycerol (SQDG) (Plouguerné et al., 2020), sulfoquinovosyl monoacylglycerol (SQMG) (Da Costa et al., 2015), and etc (Melo et al., 2015; Sanina et al., 2000), have been isolated from various marine macroalgae. In this work, the glycolipids from *Bangia fusco-purpurea*, *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp. were investigated. Among marine macroalgae, *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* contained higher content of glycolipids. Therefore, the extraction process of their glycolipids was conducted through single factor experiments (Table 1) and response surface experiments (Table 2), including some factors: the solid-to-liquid ratio, extraction temperature, time, ultrasonic power, and/or volume fraction of methanol. From single factor experiments (Fig. 1 and Fig. 2), the optimum extraction conditions of the glycolipids from *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* were obtained (Table 3). The extraction of *Bangia fusco-purpurea* extract with higher content of glycolipids was further optimized (Fig. 4). The suitable extraction conditions of the glycolipids from *Bangia fusco-purpurea* was: 1:27 g/mL, 49 °C, 98 min and 500 W, which had not been reported previously.

The existence of hydrophilic sugar group and lipophilic acyl group determines that they are amphoteric compounds. And this special amphiphilic makes they have special physiological activities. For example, glycoglycerolipids from *Sargassum vulgare* have good antifouling activity (Plouguerné et al., 2020). A galactoglycerolipid isolated from *Lobophora variegata* show significant allelopathic activity against the coral *Montastraea cavernosa* and the sponge *Agelas clathrodes* (Slattery and Lesser, 2014). Glycoglycerolipids from macroalgae present antiviral activity (Mattos et al., 2011; Wang et al., 2007), antibacterial activity (Arunkumar et al., 2005), antialgal activity (Sun et al., 2021; Sun et al., 2019), and other activities (Banskota et al., 2014; Tsai and Pan, 2012; Xiao, 2021). Among these researches mentioned-above, moisture-absorption and moisture-retention activities of glycolipids from marine macroalgae have received less attention. The researches point out that the moisture-absorption and moisture-retention mechanism of seaweed polysaccharides is (1) seaweed polysaccharides contain a large number of hydrophilic groups, such as hydroxyl and carboxyl, which can be combined with water molecules in the form of hydrogen bonds (Xu et al., 2011); (2) Algal polysaccharide molecular chain and water molecules can be crosslinked and wound in space to form a network structure (Ilekuttige et al., 2019). According to the composition of glycolipids, they should also have the moisture-absorption and moisture-retention activities. However, there was no study about the moisture-absorption and moisture-retention activities of glycolipids from marine macroalgae at present. In Fig. 3, the extracts from *Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* showed certain moisture absorption and moisturizing activity. And H₃₃, which purified from *Bangia fusco-purpurea* showed also the moisturizing activity close to that of the extract from *Bangia fusco-purpurea*, and that of glycerol, glycerin and sodium alginate in the first 42 hours. It is clear that glycolipids from *Bangia fusco-purpurea* have better moisturizing activity.

Up to now, the isolation, purification and identification of glycolipids from marine macroalgae were rarely reported (Mattos et al., 2011; Plouguerné et al., 2014; Sun et al., 2021). In view of the great application potential of glycolipids with multi activity in the medicine, food and other fields, more glycolipids from marine macroalgae need to be isolated and purified. In the present study, the extracts of *Bangia fusco-purpurea*, *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp. were selected as raw material for isolating glycolipids using liquid-liquid extraction, and preliminarily analyzed by TLC (Fig. 5) and HPLC compared with standard MGDG, SQDG and DGDG. Results showed that SQDG seems to be ubiquitous in those marine macroalgae (Table 4). The glycolipids of *Bangia fusco-purpurea* were further purified to obtain two glycolipids with arabinopyranosyloxy, hexadecanyl-1-*O*- α -D-arabinopyranosyloxy (1 $\rightarrow$ 4)- α -D-arabinopyranosyloxy (1 $\rightarrow$ 4)- α -D-arabinopyranoside and docosanyl-1-*O*- α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)-3-*O*-acetyl - α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)- α -D-arabinopyranoside (Fig. 6, Fig. 7 and Fig. 8). Those glycolipids isolated from marine macroalgae mainly contained galactose, isorhamose and glucose (Plouguerné et al., 2020; Sun et al., 2021; Wu et al., 2009). It has not been reported that the glycolipids containing arabinose have been isolated from marine macroalgae. This type of glycolipid was found only in a few corals, such as *Sinularia cervicornis* (He et al., 2002) and *Sinularia firma* (Kumar and Lakshmi, 2006). Cervicoside, an arabinomonoacyl glycolipid, exhibited cytotoxicity against human SKMG-4, Hep-G2 and CNE2 cell in vitro (He et al., 2002). H₃₃, an arabinomonoacyl glycolipid, showed moisturizing activity (Fig. 3). Surprisingly, a disaccharide (β -Gal-(1 $\rightarrow$ 3)- β -Xyl) was also obtained from *Bangia fusco-purpurea* in this study. It is reported that β -Gal-(1 $\rightarrow$ 3)- β -Xyl Used to detect intestinal lactase activity. Unfortunately, the SQDG determined by TLC and HPLC was not obtained from this macroalgae.

Marine carbohydrates are usually biodegradable and biocompatible, and their biological characteristics are helpful to discover new bioactive substances with special pharmacological characteristics. Glycolipids from marine macroalgae are also a type of marine carbohydrate, obviously, they haven't received enough attention. In *Ascophyllum nodosum* (Le Tutour et al., 1998), *Agarophyton Vermiculophyllum* (Honda et al., 2019), *Fucus vesiculosus* (Le Tutour et al., 1998), *Fucus serratus* (Le Tutour et al., 1998), and *Palmaria palmata* (Lopes et al., 2019), MGDG, DGDG and SQDG were detected (Honda et al., 2019; Le Tutour et al., 1998; Lopes et al., 2019). SQDG and DGDG were determined in *Chondrus crispus* through hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry (Melo et al., 2015). However, those glycolipids did not be isolated and purification. In future work, it is necessary to screen and isolated glycolipids from more seaweeds.

Conclusions

In the study of glycolipids from marine macroalgae such as *Capsosiphon fulvescens* (Islam et al., 2014), *Pelvetia siliquosa* (Sun et al., 2021), *Sargassum vulgare* (Plouguerné et al., 2020), *Sargassum horneri* (Ma, 2012), *Symphycladia latiuscula* (Guo et al., 2017), *Zonaria diesingiana* (Meng et al., 2008) and etc. (Lopes et al., 2014), the isolation, purification and/or identification were also concerned, but the extraction process was not concerned. In fact, the optimization of the extraction process was very

important for the preparation of glycolipids. It had been reported that the content of glycolipids in marine macroalgae was very low (Williams et al. 2007; Xia et al. 2015). Therefore, the yield of the extract contained glycolipids directly affected the content of glycolipids in the extract. In this work, the extract from *Bangia fusco-purpurea* with higher yield and the content of glycolipids was obtained by the optimized extraction process using single factor and response surface experiments. When the solid-to-liquid ratio of 1:10 g/mL, extraction temperature of 45 °C, extraction time of 120 min, ultrasonic power of 500 W, and volume fraction of methanol of 95%, the maximum yield of extract and the content of glycolipids in extract from *Bangia fusco-purpurea* were 28.1% and 116.9 µg/mL. It is noteworthy that the lowest yield of extract and the content of glycolipids in extract from *Bangia fusco-purpurea* in a single factor experiment were 17.10% and 71.12 µg/mL, respectively. The effects of the solid-to-liquid ratio, temperature, time and ultrasonic power on the *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis* were also determined using single factor experiments.

Marine macroalgae contains rich moisturizing active substances, such as polysaccharides (Ilekuttige et al, 2019; Wang et al., 2013), alginate oligosaccharide (Xu et al., 2011), mycosprine-like amino acids (MAAs) (Zhang, 2016), and etc. (Zhao et al., 2012), which are a good source of natural moisturizers. The research on moisturizing ingredients in marine macroalgae mainly focuses on seaweed polysaccharides (Chen et al., 2021; Wu et al., 2015). In this study, the glycolipids extracts from 4 species of marine macroalgae (*Bangia fusco-purpurea*, *Gracilaria lemaneiformis*, *Gracilaria* sp. and *Porphyra yezoensis*) exhibited the moisture absorption and moisturizing activities, which was better than that of sodium alginate and sorbierite, and closed to that of glycerin in a certain time. These glycolipids extracts showed good potential as moisture absorption and moisturizing agents.

Finally, isolation and purification of the glycolipids from *Bangia fusco-purpurea* were conducted through silica gel column chromatography and preparative thin layer chromatography and obtained hexadecanyl-1-*O*- α -D-arabinopyranosyloxy (1 $\rightarrow$ 4)- α -D-arabinopyranosyloxy (1 $\rightarrow$ 4)- α -D-arabinopyranoside, and docosanyl-1-*O*- α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)-3-*O*-acetyl - α -D-arabinopyranosyloxy-(1 $\rightarrow$ 4)- α -D-arabinopyranoside. This was the first report that glycolipids with arabinopyranosyloxy were found in marine macroalgae. This work also accidentally obtained a disaccharide, β -Gal-(1 $\rightarrow$ 3)- β -Xyl. In addition, the kinds of the glycolipids from *Gelidium amansii*, *Gloiopeltis furcata*, *Gracilaria lemaneiformis*, *Gracilaria* sp., *Palmaria palmata*, *Porphyra yezoensis* and *Scagassum* sp. were preliminarily determined. These macroalgae are commonly cultured on the coast of China, they can be used as the source of glycolipids to obtain some glycolipids with new structure or strong activity.

Declarations

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Conflicts of interest

The authors declared that they have no conflicts of interest to this work.

Availability of data and material

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

Ying-ying Sun conceived and designed the study. Zheng-kang Long, Li-hui Yi, Xin-yan Huang, Yang Mu and Yang-di He performed the experiments. Ying-ying Sun wrote the paper. Zheng-kang Long, Li-hui Yi and Yang Mu reviewed and edited the manuscript. All authors read and approved the manuscript.

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Figures

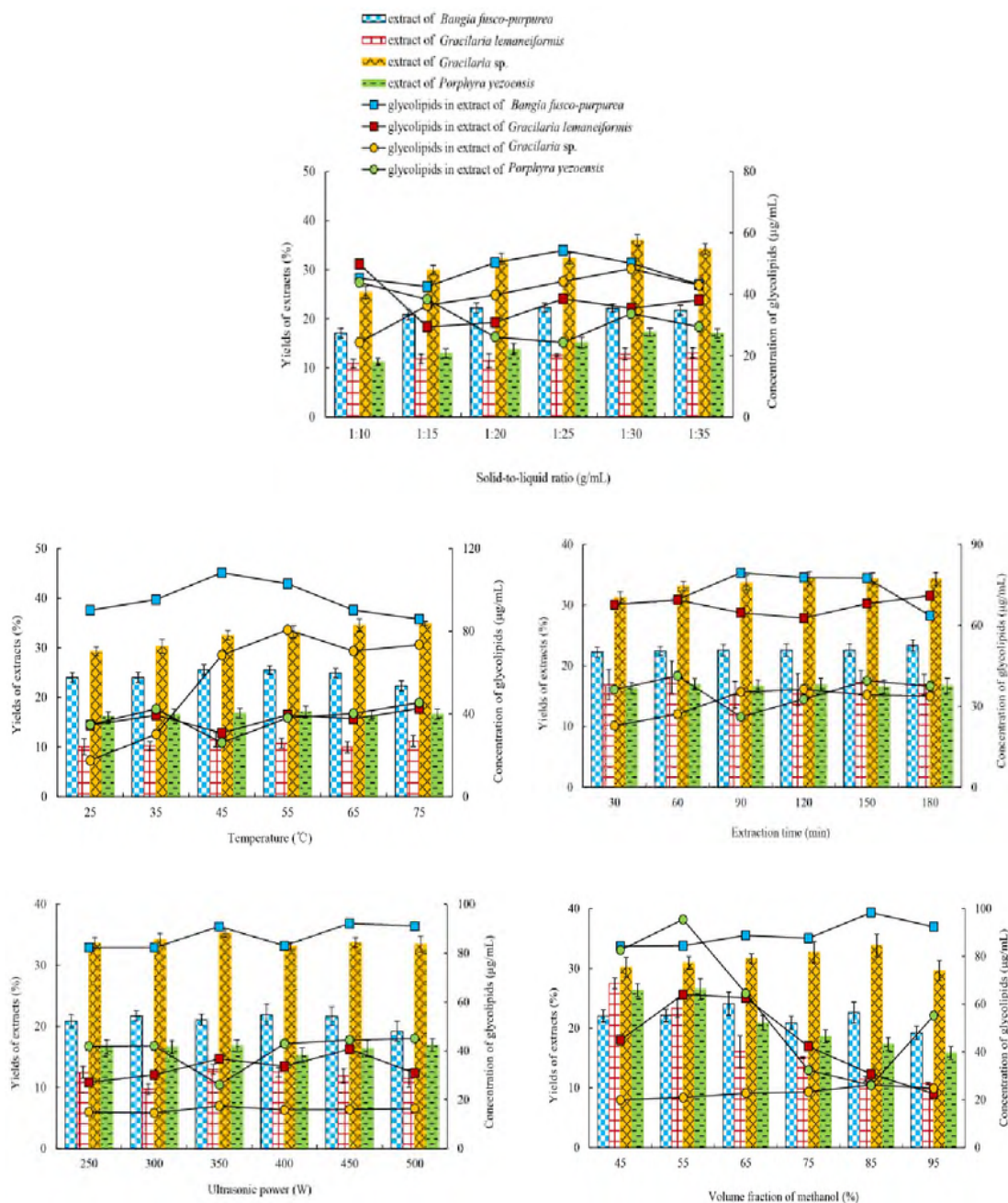


Figure 1

Effects of different factor and factor level on the yield of extract and the concentration of glycolipids in extract from 4 species of marine macroalgae in single-factor experiments

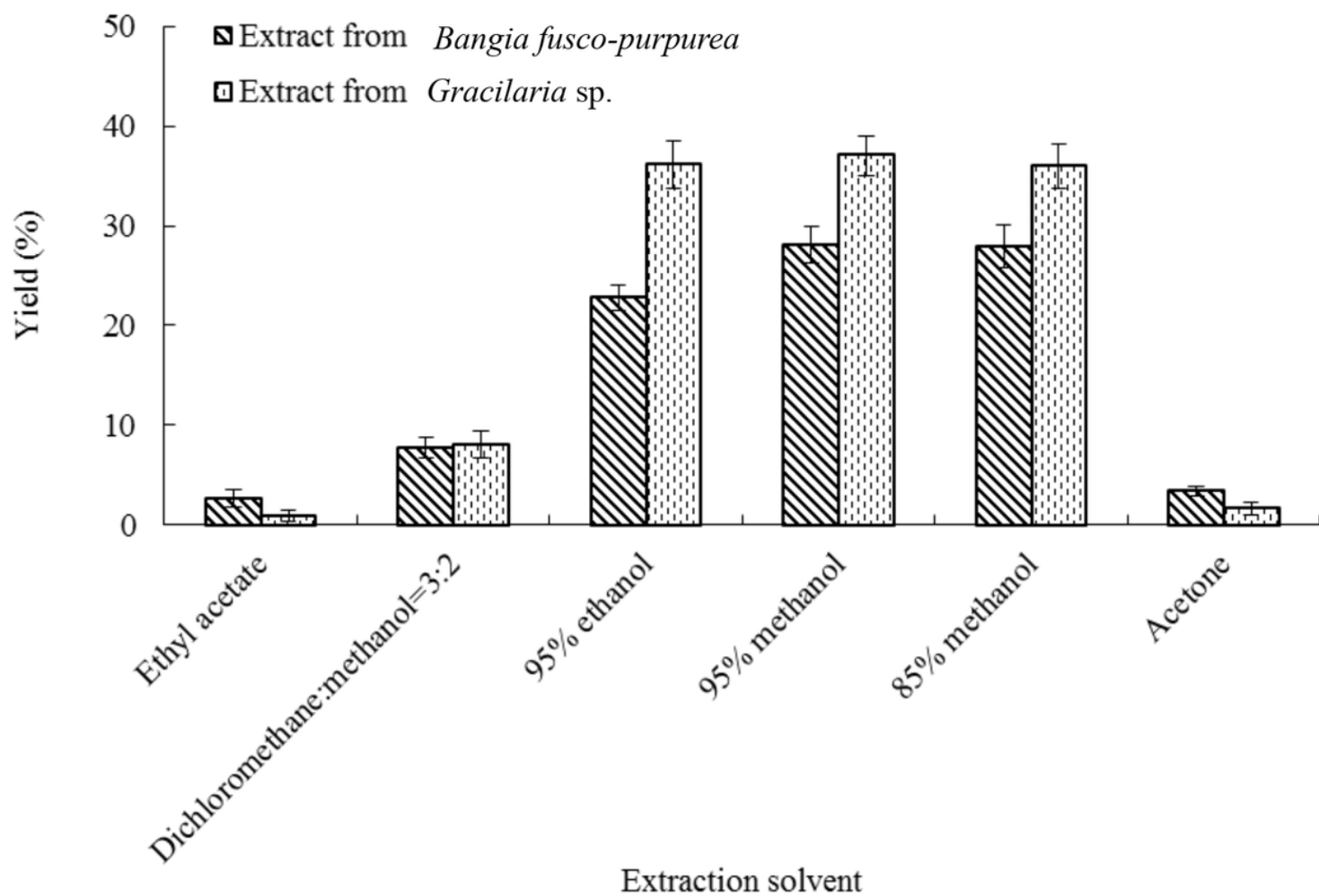


Figure 2

Effects of different solvents on the yield of extract from *Bangia fusco-purpurea* and *Gracilaria* sp.

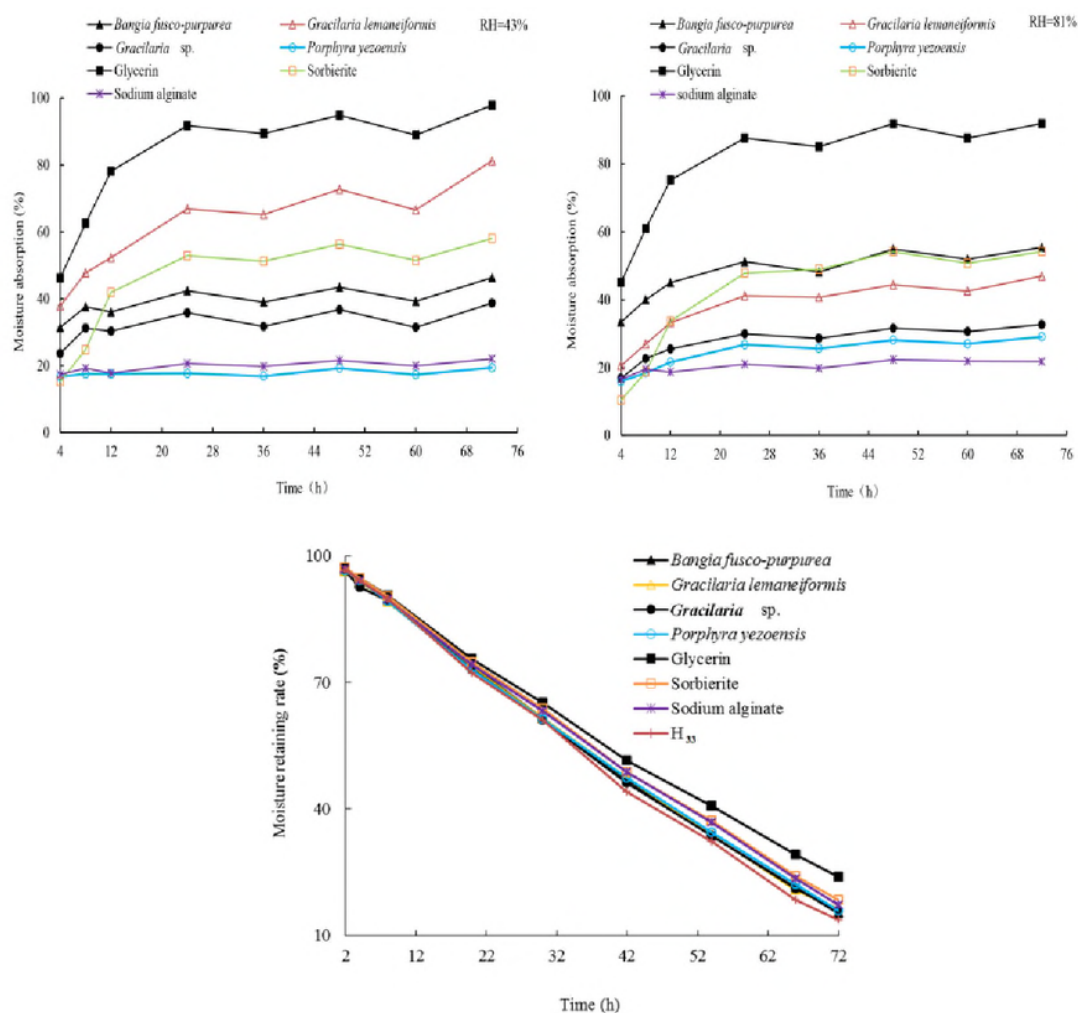
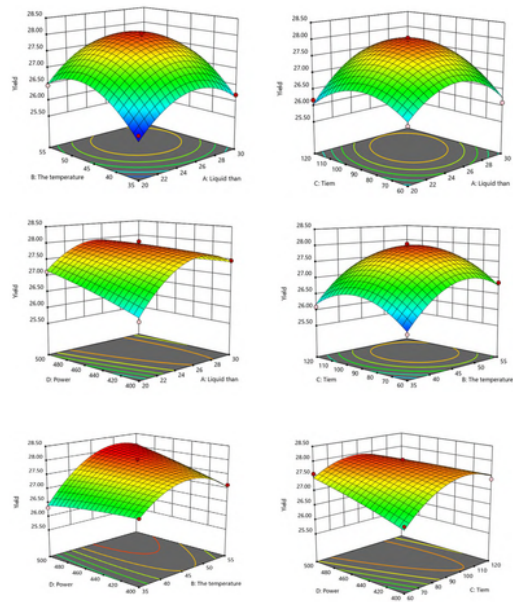
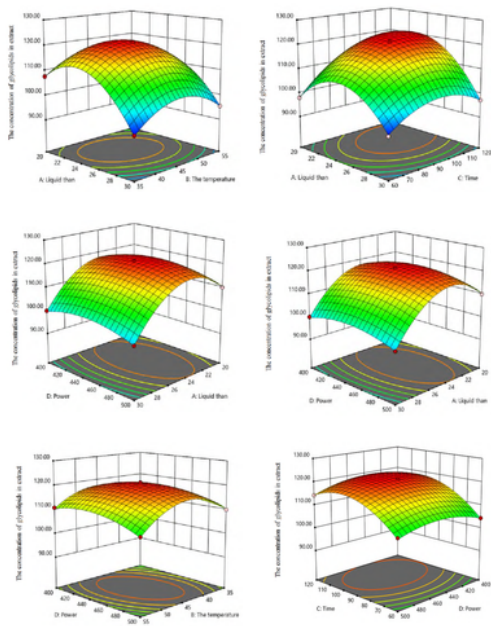


Figure 3

The Properties of moisture absorption and moisture retaining of extract from 4 species of marine macroalgae (H_{33} was purified by PTLC from from *Bangia fuso-purpurea*, and it will be described in the later separation and purification)



a



b

Figure 4

a Effects of solid-to-liquid ratio, temperature, time and ultrasonic power on the yield of extract from *Bangia fusco-purpurea* in response surface experiments b Effects of solid-to-liquid ratio, temperature, time and ultrasonic power on the concentration of glycolipids in extract from *Bangia fusco-purpurea* in response surface experiments

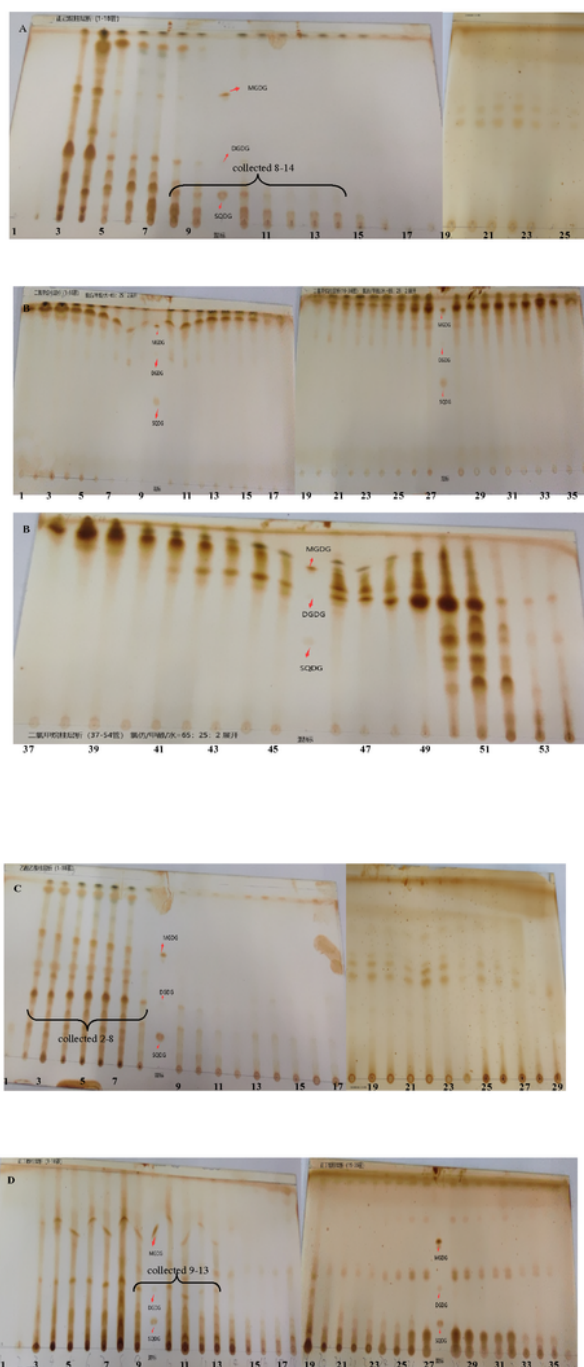


Figure 6

TLC determination of the eluents isolated from four portions from *Bangia fusco-purpurea* through silica gel column chromatography (A, B, C and D were obtained from extract from *Bangia fusco-purpurea* by liquid-liquid extraction, namely was hexane phase, Dichloromethane phase, Ethyl acetate phase and N-butanol phase, respectively. Note: MGDG, DGDG and SQDG were as a reference only, they were not used to judge whether contain the same type of glycolipids in these portions in this figure.)

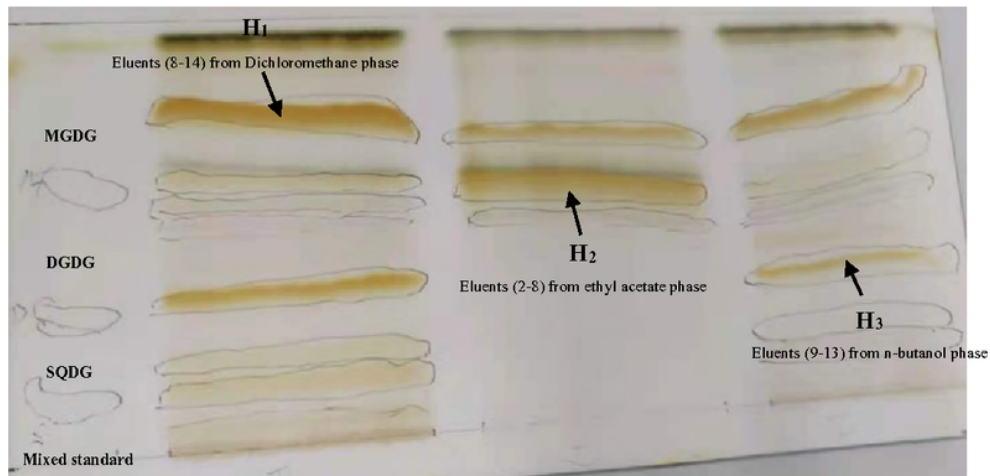


Figure 7

Preparation of thin layer chromatography of glycolipids from *Bangia fusco-purpurea*

(The strips of H₁, H₂ and H₃ were not in the same positions as that of the three standards MGDG, DGDG and SQDG. MGDG, DGDG and SQDG were as a reference only)

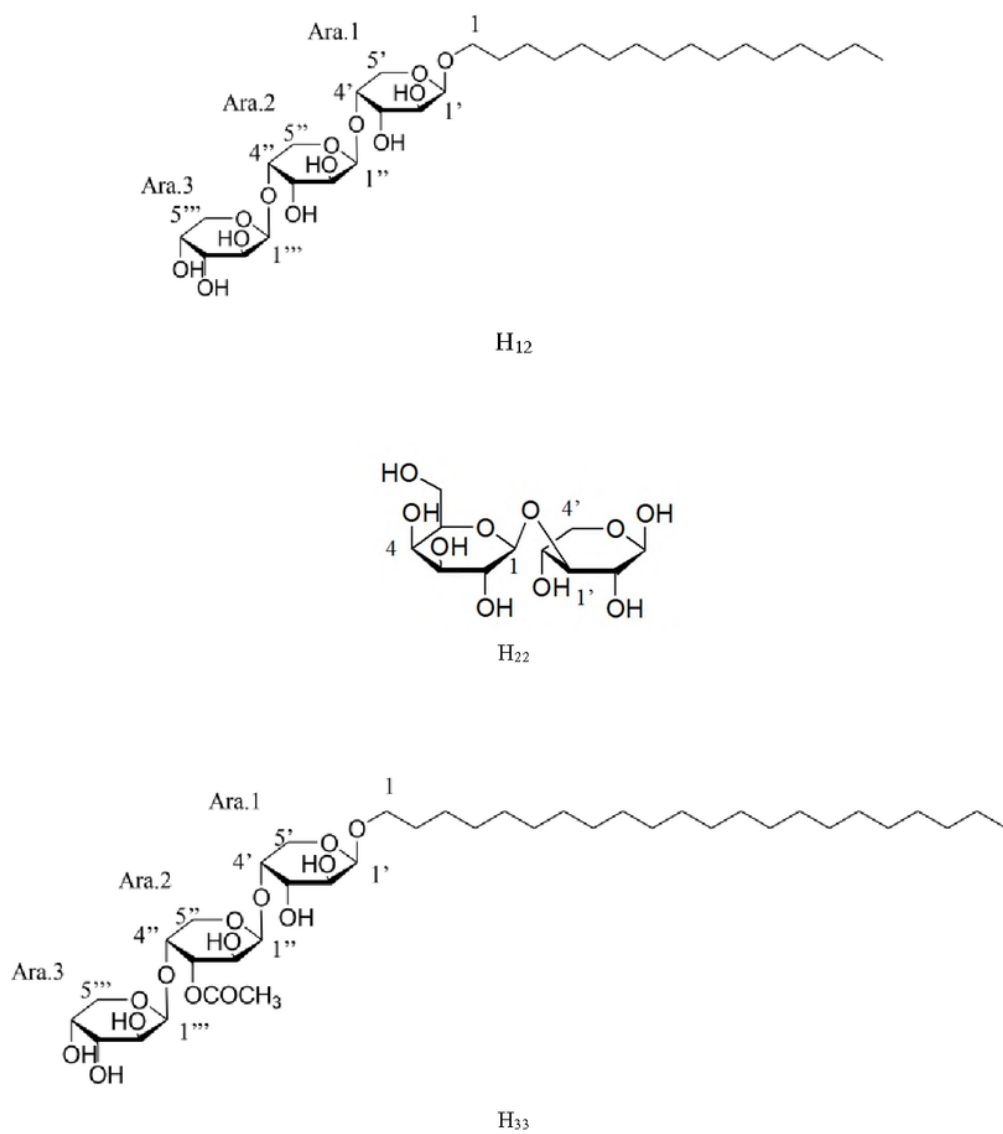


Figure 8

The structure of H₁₂, H₂₂ and H₃₃

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