Extraction and bioautographic-guided separation of antibacterial compounds from *Ulva lactuca*

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Abstract This study aimed to optimize an extraction and separation procedure to obtain a concentrated fraction with antibacterial activity from the macroalga *Ulva lactuca*. Antibacterial compounds were extracted using eight solvents, and consistent activity against *Staphylococcus aureus*, *Bacillus subtilis* and methicillin-resistant (MR) *S. aureus* was observed from a dilute (1:100, *w/v*) ethyl acetate extract. Seasonal analysis revealed that antibacterial activity was the lowest in spring/summer and the highest in autumn/winter. Bioautography was found to be a more appropriate assay compared to disc diffusion when screening crude extracts, as it separates the masking compounds from the antibacterial compounds and a direct assessment of the bands responsible for the antibacterial effect could be made. The antibacterial compounds were first separated from the crude extract using preparative thin-layer chromatography, followed by column chromatography to obtain a semi-pure sub-fraction. Using this approach, the antibacterial compounds were successfully concentrated from a crude extract (300 μg) to semi-pure fractions (6 μg) in which antibacterial activities were greatly enhanced. This study also revealed that prolonged storage (9 months) under a nitrogen atmosphere at −20°C resulted in a considerable increase in antibacterial activity. This is the first report of seasonal assessment of antibacterial compounds from seaweeds collected in Ireland. In addition, an antibacterial fraction was successfully isolated from *U. lactuca* which exhibited potent anti-MR *S. aureus* activity.

Keywords MR *S. aureus* · Disc diffusion · Seasonal variation · Seaweeds · Ireland · Column chromatography

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Introduction

Bacterial infections are a common problem in hospitals and clinical settings worldwide. Typically, treatment of such infections is by administration of antibiotics; however, indiscriminate use of antibiotics has led to increased bacterial resistance (Levy 2001). Methicillin-resistant (MR) *Staphylococcus aureus* infections alone are responsible for the death of more people annually in the USA and in Europe than HIV and AIDS and are, therefore, a serious global issue (Finch and Hunter 2006; Kelland and Hirschler 2011). The emergence of antibiotic-resistant bacteria has resulted in the need to develop novel alternatives to current antibiotics. In addition, there is increased public awareness of the overuse of antibiotics and an increasing trend towards using medicinal plants as effective alternatives (Ahmad and Aqil 2009; Arora et al. 2010). Extensive studies have been conducted to examine antibiotics derived from natural products with a view to identifying novel compounds with efficacy in treating infections, particularly those caused by antibiotic-resistant bacteria (Ravikumar et al. 2002; Amghalia et al. 2009; Yuvaraj et al. 2011).

Studies on the antibacterial activity in seaweeds dates back to 1954 for a single seaweed, the brown *Ascophyllum nodosum*, to recently, where a wide range of seaweeds including brown, red and green seaweeds were screened (Vacca and Walsh 1954; Reichelt and Borowitzka 1984; Chanda et al. 2011). Targets have included both Gram-positive and Gram-negative bacteria and pathogenic as well as non-pathogenic strains (Vacca and Walsh 1954; Reichelt and Borowitzka 1984; Allmendinger et al. 2010; Cox et al. 2010; Chanda et al. 2011).

It has been suggested that compounds with antibacterial activity produced by seaweeds are secondary metabolites (Elsie et al. 2011). Production of these compounds is affected by plant growth which, in turn, is affected by environmental factors such as water temperature, irradiation and presence of metal ions (Nishihara et al. 2004; Kakita and Kamishima 2006). It is postulated that the production of antibacterial compounds may increase when seaweeds are exposed to environmental stresses, or to avoid bacterial attack if the plant is damaged due to environmental conditions or herbivore grazing (Vlachos et al. 2001; Figueiredo et al. 2008). A better understanding of the conditions associated with antibacterial compound production in seaweeds would enable harvesting at the most appropriate season. Furthermore, it may be possible to artificially stress the species into producing higher levels of secondary metabolites.

A number of techniques are used to assess the antibacterial capacity of compounds, namely disc diffusion, bioautography and broth dilution (Rios et al. 1988). Both disc diffusion and bioautography are qualitative measurements that indicate presence or absence of antibacterial agents, while the broth dilution method is a quantitative assay used for assessment of the minimum inhibitory concentration (MIC) (Rios et al. 1988; Silva et al. 2005; Valgas et al. 2007; Wikler et al. 2009a). The disc diffusion assay is simple and practical and is the most commonly used method for antimicrobial screening recommended by the Clinical and Laboratory Standards Institute (CLSI) (Wikler et al. 2009b) while bioautography facilitates a more comprehensive investigation of the extract through a combination of sub-fractionation coupled with visualization of antibacterial efficacy of the fractions (Martini and Eloff 1998; Smith et al. 2007). *Ulva lactuca* (Ulvophyceae; Ulvaceae), commonly known as 'sea lettuce', is found in abundance in Ireland and can be a nuisance in nutrient-rich areas such as harbours or ports. *U. lactuca* has been known to contain compounds with antibacterial activity against a wide range of bacteria (Flodin et al. 1999; Awad 2000; Kumar and Rengasamy 2000; Kandhasamy and Arunachalam 2008). For example, Kim et al. (2007) reported that a crude diethyl ether extract of *U. lactuca* collected from the coastal area of Busan,

South Korea was effective against MR *S. aureus* at a concentration of 12 µg mL^{-1}. However, no further separation or identification of the anti-MR *S. aureus* compounds was performed.

In the present study, crude extracts of *U. lactuca* were generated using a series of solvents with different polarities in order to identify the most suitable solvent for extraction of antibacterial compounds. This is the first study conducted on *U. lactuca* which attempts to separate, isolate and study the anti-MR *S. aureus* compounds in the crude extract. Additionally, this study aimed to determine the seasonal effects, if any, on antibacterial production in *U. lactuca* and to compare the efficacy of disc diffusion and bioautography in assessment of antibacterial activity in the crude extract. Lastly, the effect of prolonged storage at −20°C on antibacterial activity of the crude seaweed extract was also investigated.

Materials and methods

Ulva lactuca was harvested from the intertidal zone of Baginbun Head, Wexford, Ireland (52°10′23″ N, 6°49′26″ W) at low tide, on September 2009 and March 2010. The collected seaweeds were washed with distilled deionized water (SG Water, Germany) to remove sand and epiphytes and frozen at −20°C overnight. The frozen samples were subsequently freeze-dried, blended, sieved and stored under a nitrogen atmosphere at −20°C for further analysis.

Initially, the dried blended seaweed powder was extracted using different solvents (1:100, *w/v*) with decreasing polarities: *n*-hexane, chloroform, ethyl acetate, dichloromethane, acetone, ethanol, methanol and water in order to select the most suitable solvent for extraction of antibacterial compounds. All chemicals used were HPLC grade. The seaweeds were extracted on a shaker for 2 h at room temperature, and solvents were removed by rotary evaporation under vacuum. The dried extracts were stored under a nitrogen atmosphere at −20°C until further analysis. The initial antibacterial analyses of the crude extracts were performed using samples harvested in September 2009 and March 2010.

Preparation of bacterial cultures for antibacterial testing The indicator bacteria used were MR *S. aureus* W73365, *S. aureus* DPC 5246, *Listeria innocua* DPC3572, *Bacillus subtilis* ATCC 6633 and *Enterococcus faecalis* DPC 4928 (all Gram positive) and *Cronobacter sakazakii* ATCC 12868, *Pseudomonas aeruginosa* PA01, *Escherichia coli* DSMZ 10720 and *Salmonella typhimurium* LT2 (all Gram negative). Each strain was stocked in sterile broth containing 40% glycerol and stored at −20°C. For antibacterial assessment, each strain was inoculated (1%)

into brain heart infusion broth (BHI, Oxoid, England) and incubated overnight at 37°C with the exception of *B. subtilis* and *P. aeruginosa* which were incubated at 28°C with *B. subtilis* agitated at 200 rpm.

Antibacterial screening using the disc diffusion assay The antibacterial activity of each crude extract was tested using a disc diffusion assay, based on the CLSI method (Wikler et al. 2009b). Briefly, overnight cultures were washed twice with maximum recovery diluent and adjusted to an optical density equivalent to that of a 0.05 McFarland Standard or 10^7–10^8 colony forming units per mL (CFU mL^{-1}) using a spectrophotometer. The adjusted culture was used within 15 min. Reconstituted crude extract (1 mg) was loaded onto 6-mm blank paper discs (Oxoid, England), and 100 μL of the respective solvents was loaded as negative controls. Chloramphenicol antibiotic discs (10 μg, Oxoid, UK) were used as a positive control. The discs were placed onto Mueller Hinton agar (MHA) (Becton Dickinson and Company, France) plates which had been swabbed with the adjusted bacterial suspension, and the plates were refrigerated at 5°C for 5 h to allow diffusion of the extracts into the agar before incubating at 37°C for 18–20 h. Antibacterial activity was observed as clear inhibition zones around the paper discs, and the diameter of inhibition was measured. The antibacterial activities of extracts generated with solvents of different polarities were determined in duplicate.

Comparison of the disc diffusion assay with bioautography for assessment of antibacterial activity of crude extracts A comparative study to examine efficacy of the disc diffusion assay in comparison to bioautography for assessment of the antibacterial activity of crude algal extracts was performed using an ethyl acetate extract of *U. lactuca* collected from May 2010 to May 2011. Bioautography was performed based on the method of Smith et al. (2007) with minor modifications. Briefly, 30 μL (300 μg) of the stock extract (10 mg mL^{-1}) was spotted onto silica gel 60F$_{254}$ aluminium thin-layer chromatography (TLC) plates (Merck, Germany), together with 30 μL of ethyl acetate as a negative control and 0.5 μg of novobiocin (Sigma, Ireland) as a positive control. The TLC plates were developed in a suitable solvent system and were left to dry for at least 30 min, aseptically, to remove the mobile phase. The developed TLC plates were transferred aseptically to a sterile Petri dish. A blank TLC plate developed with the mobile phase was also used as a negative control. The sterile TLC plates were overlaid with 15 mL of molten MHA seeded with an overnight culture of the indicator bacterium (150 μL of *S. aureus* or MR *S. aureus*; 450 μL of *B. subtilis*). The plates were incubated at 37°C for 18–20 h. Following incubation, the plates were sprayed with 2,3,5-triphenyltetrazolium

chloride (MTT; 2.5 mg mL^{-1}) and were incubated at 37°C for 3 h prior to measuring the inhibition zones. Metabolically active bacteria convert the MTT into formazan dye, revealing the inhibition zone as a colourless region against a red background. The area of the inhibition zones was calculated as $\pi \times r_1 \times r_2$, where r_1 is the vertical radius and r_2 is the horizontal radius of the inhibition zone (Gende et al. 2008). The disc diffusion assay was performed as outlined above. Extracts were analysed in triplicate.

Effect of season on the production of antibacterial compounds in U. lactuca The effect of season on antibacterial activity of the ethyl acetate extracts against *S. aureus*, MR *S. aureus* and *B. subtilis* was investigated with seaweeds collected monthly between May 2010 and May 2011 using both the disc diffusion assay and bioautography as previously described. Extracts were stored under a nitrogen atmosphere at −20°C and were tested for activity using bioautography as previously described. Extracts were analysed in triplicate.

Separation of antibacterial compound(s) from crude extracts Dried powdered *U. lactuca* (110 g) collected in July 2010 was extracted with ethyl acetate as previously outlined, yielding 0.743 g (0.68%) of crude extract. Initially, the concentrated crude extract was purified using pre-conditioned preparative TLC (Analtech, Lennox Laboratory, Dublin, Ireland). The extract was applied across the preparative TLC plate, and the plate was developed. Four fractions were visible and the active fraction, fraction 2 ('F2') (75.6 mg), determined by bioautography, was carefully scraped from the preparative TLC plate, re-extracted with ethyl acetate and filtered to remove silica.

The active 'F2' fraction was further purified using column chromatography packed with silica gel 60 (230–400 mesh, Fluka Analytical) using increasing polarity of 2:8 to 4:6 (*v/v*) ethyl acetate, hexane and methanol mobile phase. Samples were collected in 10 mL volumes and ten sub-fractions were pooled based on bioautography using *B. subtilis* as an indicator. The antibacterial activities of the ten sub-fractions were confirmed against *S. aureus* and MR *S. aureus* using bioautography.

HPLC analysis of purified sub-fractions collected from column chromatographic separation The dried sub-fractions (ten) collected from the chromatographic separation were reconstituted and analysed by high-performance liquid chromatography (HPLC). The HPLC system used was an Agilent 1200 series equipped with an Agilent 1200 series binary pump SL, an Agilent 1200 series G1316B SL temperature-controlled column oven, a micro-vacuum degasser and a photodiode array (PDA) detector, controlled

by EZChrom software. Separation was achieved using a silica column (Nucleosil 100 silica 5 μm, 250×4.6 mm, Supelco, Ireland) with a mobile phase consisting of 20:80 (v/v) tetrahydrofuran/hexane at a flow rate of 1 mL min^{-1}, and pressure was maintained at 50 psi. Eluent analysis was carried out using a PDA detector, with detection from 210 to 655 nm.

Statistical analysis Seasonal data on antibacterial activity of *U. lactuca* were first analysed with two-way ANOVA. Initial analysis of the data suggested that the two variables—type of bacteria (MR *S. aureus*, *S. aureus* and *B. subtilis*) and collection month—influenced the antibacterial activity. As a result, a repeated measures one-way ANOVA was performed with 36 levels of factors, one for each month and bacterial type combination, followed by a post hoc analysis using Tukey's multiple comparison test. These analyses were performed using GraphPad Prism version 5 for Windows using a 5% statistical significance level ($P<0.05$).

Results

Antibacterial screening of seaweed extracts using the disc diffusion assay

Ulva lactuca was extracted using eight solvents, with decreasing polarity. Extracts were initially screened against a wide range of indicator bacteria, namely *S. aureus* (including MR *S. aureus*), *B. subtilis*, *E. coli*, *S. typhimurium*, *L. innocua*, *C. sakazakii*, *E. faecalis* and *P. aeruginosa*, using the disc diffusion assay. Of the samples from September 2009, the ethyl acetate extract had the greatest activity and also the broadest spectrum of inhibition as it inhibited *S. aureus*, *B. subtilis* and MR *S. aureus*. Methanolic extracts had low activity against MR *S. aureus* and extracts generated using water, ethanol and chloroform slightly inhibited the growth of *B. subtilis* (Table 1). A second sample of *U. lactuca* was collected 6 months later, and extracts were generated using the same eight solvents and tested for activity against the same indicator bacteria. These samples had different antibacterial profiles compared to the September 2009 samples (Table 1). For example, antibacterial activity was not observed from the acetone and dichloromethane extracts in the September 2009 samples, whereas in March 2010, weak activity was observed from the acetone extract against *B. subtilis* and MR *S. aureus* and from the dichloromethane extract against *B. subtilis*. Activity against *S. aureus* and *B. subtilis* was observed from the ethanol extract in September 2009 but not in March 2010, while the methanol extract was active against MR *S. aureus* in the September 2009 samples but was inactive against MR *S. aureus* but active against *S. aureus* in the March 2010 samples.

The hexane extracts generated from the March 2010 samples displayed strong activity against *S. aureus*, *B. subtilis*, MR *S. aureus* and *E. faecalis*, but activity was not

Table 1 Antibacterial activity of *U. lactuca* extracts generated with different organic solvents as determined by disc diffusion assay

Solvent	Antibacterial activity									
	September 2009					March 2010				
	% Yield	MR *S. aureus*	*S. aureus*	*B. subtilis*	*E. faecalis*	% Yield	MR *S. aureus*	*S. aureus*	*B. subtilis*	*E. faecalis*
Water	16.3	−	−	+	−	28.6	−	−	−	−
Methanol	10.6	+	−	−	−	6.86	−	+	−	
Ethanol	1.64	−	+	+	−	1.08	−	−	−	−
Acetone	0.68	−	−	−	−	1.20	+	−	+	−
Dichloromethane	0.01	−	−	−	−	0.53	−	−	++	−
Ethyl acetate	0.11	++	++++	++++	−	0.49	+	+	+++	−
Chloroform	0.28	−	−	+	+	0.55	+	−	++	−
Hexane	0.18	−	−	−	−	0.27	+	+++	++++	+
Positive control[a]	−	++++	++++	++++	++++	−	++++	++++	++++	++++
Negative control[b]	−	−	−	−	−	−	−	−	−	−

Inhibition zones are reported as diameters of clear zones (including 6 mm diameter of blank discs), analysed in duplicate. No antibacterial activity was observed against *E. coli*, *S. typhimurium*, *L. innocua*, *C. sakazakii* or *P. aeruginosa*

− no activity, + inhibition zone of 6–7 mm, ++ inhibition zone of 7.1–8 mm, +++ zone of 8.1–10 mm, ++++ inhibition zone >10 mm

[a] Chloramphenicol antibiotic disc (10 μg)

[b] Negative control was 100 μL of each of the respective solvents

present in the September 2009 samples. The ethyl acetate extract was the only extract that demonstrated consistent inhibition against *S. aureus*, *B. subtilis* and MR *S. aureus* at both time points. None of the extracts were active against any of the Gram-negative bacteria tested, i.e. *E. coli*, *S. typhimurium*, *P. aeruginosa* and *C. sakazakii*.

The percentage yield of the crude seaweed extracts also varied (Table 1). In general, the yield was higher for the March 2010 samples compared to the September 2009 samples for all solvents except methanol and ethanol. However, no relationship between the percentage yield and the antibacterial activity was observed. For example, the highest yield was obtained when *U. lactuca* was extracted using water; however, activity was only observed against *B. subtilis* and only in the September 2009, while the hexane extract from the March 2010 samples had the lowest yield but had the broadest spectrum of activity. The ethyl acetate extract displayed consistent antibacterial activity and was therefore chosen for further study.

Comparison of the disc diffusion assay with bioautography for assessment of antibacterial activity of crude extracts

Production of antibacterial compounds was monitored in ethyl acetate extracts from *U. lactuca* collected monthly from May 2010 to May 2011 using the standard disc diffusion assay as well as bioautography. The sensitivity of both assays in assessing antibacterial activity against MR *S. aureus* in the crude extract was compared (Fig. 1). Results indicated that antibacterial activity was consistently evident when assessed using bioautography, whereas activity was not consistently observed using the disc diffusion assay. Activity from these extracts was also examined against *S. aureus* and *B. subtilis*, and a similar trend was seen (data

not shown). The antibacterial activity observed using bioautography was in the colourless region with R_f values between 0.5 and 0.7 (data not shown), indicating that all samples contained the same active compounds or similar classes of compounds (Fig. 1).

Effect of season on the production of antibacterial compounds in *U. lactuca*

As bioautography proved to be a more sensitive method to examine antibacterial activity in crude extracts of *U. lactuca*, it was used to examine the effect of seasonality on antibacterial production (Fig. 2). There was no difference in the anti-MR *S. aureus* activity for samples collected in May 2010, June 2010, July 2010, August 2010, March 2011 and May 2011 ($P<0.05$) (Fig. 2a). Generally, the antibacterial activity against all three indicator bacteria increased from summer/autumn, peaked in winter and subsequently decreased in spring/summer (Fig. 2). There was no significant difference in the antibacterial activity against all three indicator bacteria between the May 2010 and the corresponding May 2011 samples. No sample was collected in December 2010 due to adverse weather conditions.

In order to examine the effect of prolonged storage on the anti-MR *S. aureus* activity of the extracts, all extracts were stored under a nitrogen atmosphere at −20°C and were re-examined after several months of storage (Fig. 3). Interestingly, an increase in anti-MR *S. aureus* activity was observed with increased storage time (Fig. 3). No change in activity was observed for samples that were stored for less than 3 months, but a greater than 50% increase in antibacterial activity was observed in samples that were stored for more than 5 months. Antibacterial activity remained constant in samples stored from 5 to 8 months and increased more than 170% when stored for 9 months (280 days).

Separation of antibacterial compound(s) from crude *U. lactuca* extracts

The first step in purifying the crude seaweed extract was to use preparative TLC to separate the antibacterial compounds from the non-active, coloured compounds (Fig. 1). Four fractions were collected from the preparative TLC plates, and the activities of these fractions were assessed and compared using bioautography (Fig. 4). Even though the samples used for separation were collected in different months (May, July, September and October 2010), generally, F2 was consistently found to have the greatest antibacterial activity, with the exception of samples collected in September 2010 tested against *S. aureus* (Fig. 4b). Higher antibacterial activity was observed from F2 com-
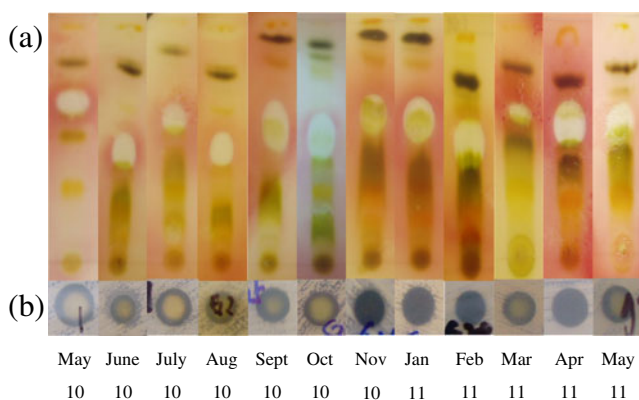

(a)

(b)

| May | June | July | Aug | Sept | Oct | Nov | Jan | Feb | Mar | Apr | May |
| 10 | 10 | 10 | 10 | 10 | 10 | 10 | 11 | 11 | 11 | 11 | 11 |

Fig. 1 Comparison of antibacterial activity of a crude ethyl acetate extract from *U. lactuca* using *a* bioautography (300 μg/spot) and *b* the standard disc diffusion assay (1 mg/disc). In both cases, *S. aureus*, MR *S. aureus* and *B. subtilis* were used as indicator organisms. Seaweed samples were harvested and analysed between May 2010 and May 2011
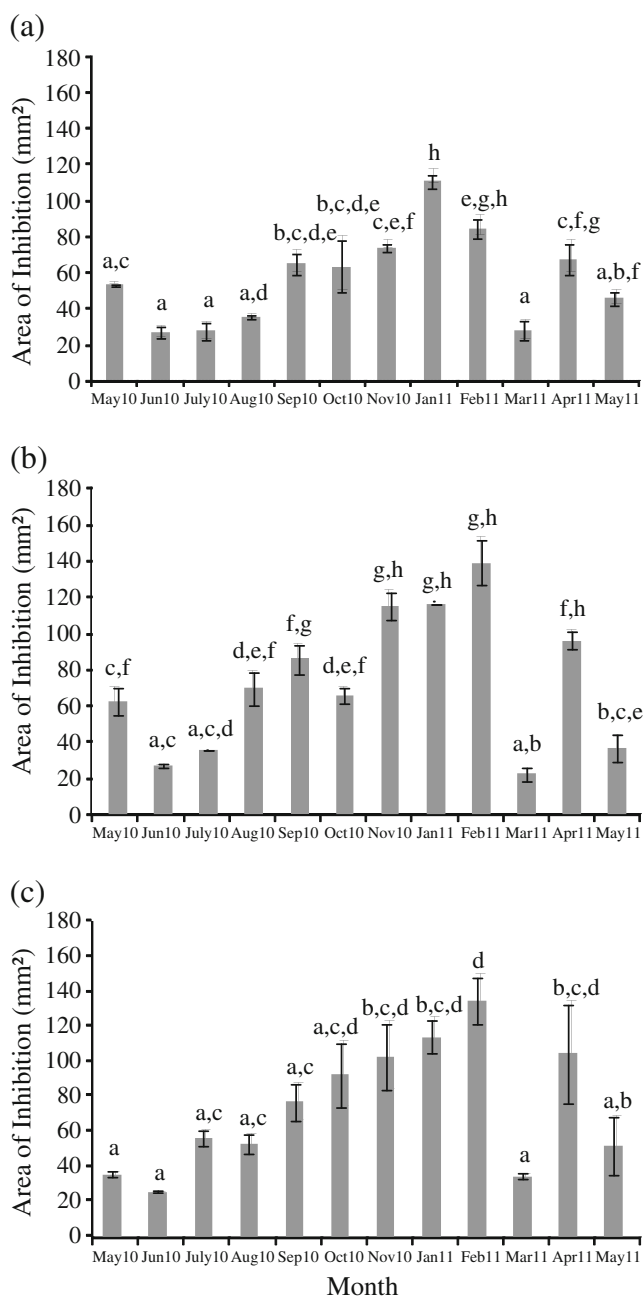
(a)

(b)

(c)

Fig. 2 Antibacterial activity of a crude *U. lactuca* extract against **a** MR *S. aureus*, **b** *S. aureus* and **c** *B. subtilis* using bioautography. Data are mean of triplicate analyses±SE. *Bars that do not share a common letter are statistically different* (*P*<0.05, one-way ANOVA and post hoc analysis using Tukey's multiple comparison test). SEM for MR *S. aureus*, *S. aureus* and *B. subtilis* were 5.04, 5.74 and 10.8, respectively. The overall *P* value for the three bacteria was <0.0001

Fig. 3 Effect of storage of the crude *U. lactuca* extract at −20°C on activity against MR *S. aureus* using bioautography. Data are the means of duplicate analyses±range

and September 2010 samples (Fig. 4c). However, fraction 3 (F3) and fraction 4 (F4) also exhibited antibacterial activity, indicating that they may have traces of antibacterial compounds also.

The antibacterial compounds contained in F2 were further fractionated using column chromatography. The initial mobile phase successfully removed the non-active, coloured compounds, and the antibacterial compounds were eluted when the polarity of the mobile phase was increased. Finally, additional mobile phases were used to elute the strongly bound compounds.

A total of ten sub-fractions were pooled from separation of F2 based on their TLC profiles, and the activity of each sub-fraction was tested against *S. aureus*, *B. subtilis* and MR *S. aureus* using bioautography to confirm the presence of antibacterial compounds. The majority of the antibacterial compounds were collected in sub-fraction (SF) 4 with trace amounts in SF3, SF5 and SF6 (Table 2). SF1, SF7 and SF8 were found to have some antibacterial compounds, but the antibacterial profile was different from the other fractions. Antibacterial activity was absent from SF2, SF9 and SF10. The activity of SF4 was re-evaluated using bioautography, and this revealed that 6 μg was sufficient to reduce the growth of MR *S. aureus* and *B. subtilis* and 10 μg was sufficient to inhibit the growth of MR *S. aureus* (Fig. 5).

HPLC analysis of purified sub-fractions collected from column chromatographic separation

All ten sub-fractions were analysed using HPLC with PDA detection. A peak was evident at 2-min retention which corresponded well with the antibacterial activity observed for the ten sub-fractions (Table 2) using bioautography. Based on the HPLC profile in Fig. 6, it was found that SF4

pared to the crude extract, indicating that F2 contained most of the antibacterial compounds, as the concentration used for bioautography testing was the same (300 μg). No activity was observed from fraction 1 (F1), except for a slight inhibition observed against MR *S. aureus* in the July 2010 sample (Fig. 4a) and against *B. subtilis* for the July
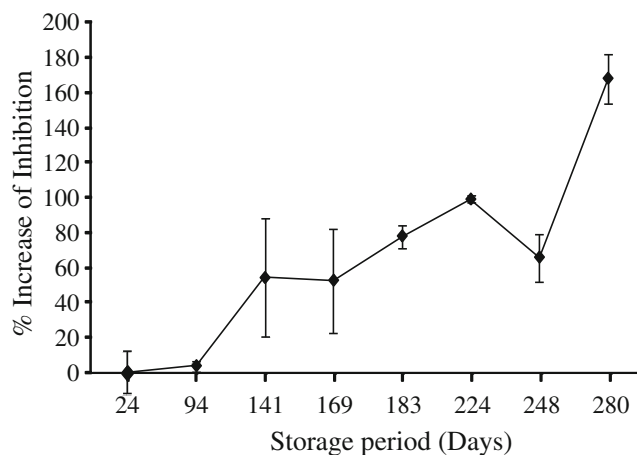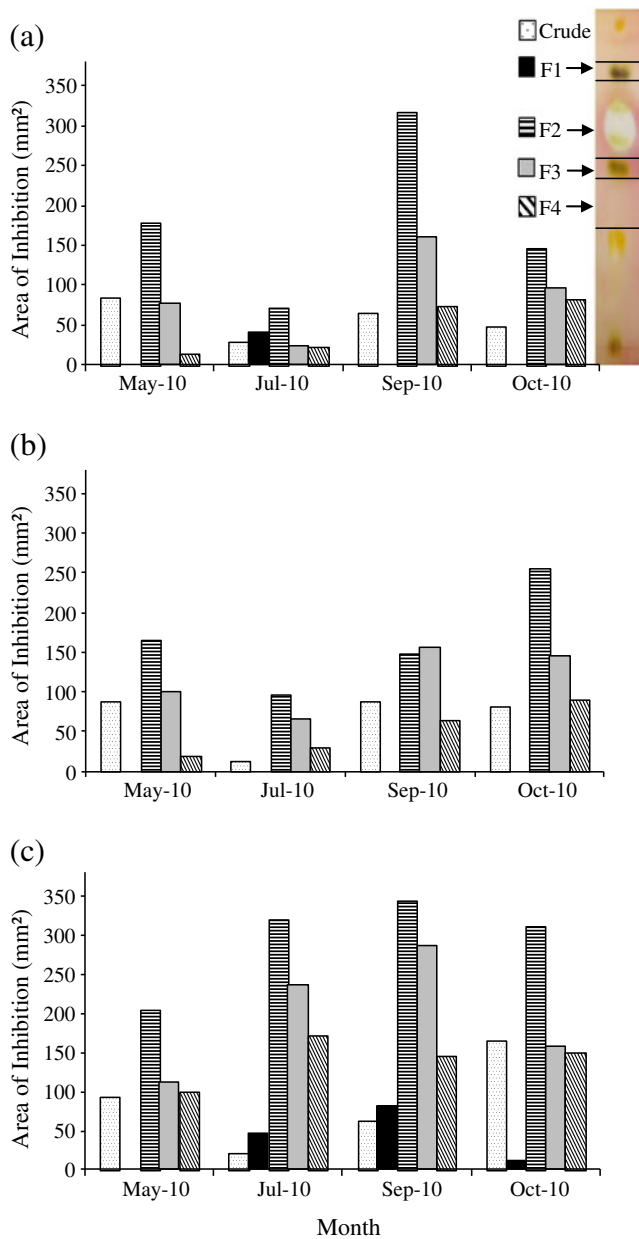
Fig. 4 Comparison of the areas of inhibition against **a** MR *S. aureus*, **b** *S. aureus* and **c** *B. subtilis* for the crude *U. lactuca* extract and preparative TLC fractions. Data were obtained from samples collected at different months using bioautography. The different fractions (F1, F2, F3, F4) collected from preparative TLC separation are shown in the *upper right side* of the diagram

had the highest antibacterial peak (97% of the total peak area), with two minor peaks eluting at approximately 5 min.

Discussion

Many studies have investigated the antibacterial activity of crude seaweed extracts (Cox et al. 2010; Gupta et al. 2010), but few studies have purified compounds from crude

seaweed extracts with activities similar to those found in this study. The main focus of the present study was to examine the suitability of a number of solvents for extraction of antibacterial compounds from *U. lactuca*, to investigate the seasonality of antibacterial production and, finally, to separate the antibacterial compounds from the crude extract. The initial concentration of the crude extracts used in this study was relatively dilute (1:100, *w/v*) compared to previous studies which used more concentrated crude extracts, such as 1:2 (*w/v*) (Kolanjinathan and Stella 2009), 1:10 (*w/v*) (Manilal et al. 2009), 1:40 (*w/v*) (Ibtissam et al. 2009) or 1:50 (*w/v*) (Taskin et al. 2007). Antibacterial compounds present in crude extracts of *U. lactuca* were relatively potent, as activity was evident even from dilute crude extracts. For example, Kolanjinathan and Stella (2009) used concentrated *U. lactuca* extracts (1:2, *w/v*) and reported high inhibitory activity against *S. aureus* and *E. coli*. On the other hand, Ibtissam et al. (2009) who used more dilute samples (1:40, *w/v*) reported large inhibition zones (16 mm) against *E. coli*, *S. aureus* and *E. faecalis*. Interestingly, in the present study, activity against *S. aureus* was observed even when a dilute crude extract (1:100, *w/v*) was used, indicating that even small amounts of the antibacterial compound(s) from *U. lactuca* can exhibit an antibacterial effect. However, the lack of inhibitory activity against *E. coli* and *E. faecalis* from the methanolic extract may be attributable to the lower concentration of crude extract used and/or to the presence of different bioactive compounds to those reported in the literature. Apart from the concentration factor, the production of antibacterial compounds in seaweeds may be affected by harvest location. For example, methanolic extracts of *U. lactuca* collected from New York, USA did not exhibit antibacterial activity against *E. coli*, even when a more concentrated extract (1:10, *w/v*) was used (Lustigman and Brown 1991), but extracts generated from the same species (*U. lactuca*) collected in Morocco did display antibacterial activity even though a lower concentration of extract (1:40, *w/v*) was used (Ibtissam et al. 2009). These differences in activities may be attributed to different chemical compositions of the seaweeds which may be influenced by geographical and environmental factors (Figueiredo et al. 2008).

A broader spectrum of antibacterial activity was observed from the hexane extract of the March 2010 samples, but this was not chosen for further study because the activity was inconsistent. Ibtissam et al. (2009) also reported a similar pattern where activity against *E. coli*, *S. aureus* and *E. faecalis* was seen in an initial study with *U. lactuca* collected in August 2003, but not from samples collected 3 years later, in February 2007. This suggests that the compounds responsible for the antibacterial activity in the hexane extract were also season-dependent. This was also the case for the other

Table 2 Comparison of the area of the bioactive peak from HPLC analysis in Fig. 6 with the area of inhibition of the different sub-fractions against different indicator bacteria using the bioautography assay

Sub-fraction	Retention time (min)	Area of peak from HPLC (mAu×s)	Area of inhibition (mm^2)		
			MR *S. aureus*	*S. aureus*	*B. subtilis*
SF1	2.020	1,553	23.6	ND	127
SF2	ND	ND	ND	ND	ND
SF3	2.010	530	80.2	55.0	55.0
SF4	2.044	2,714	409	280	332
SF5	2.025	290	86.4	236	77.0
SF6	2.022	325	38.5	94.3	28.3
SF7	2.027	232	3.1	27.5	ND
SF8	2.021	382	ND	28.3	7.1
SF9	2.028	217	ND	ND	ND
SF10	2.027	250	ND	ND	ND

solvent extracts from the September 2009 and March 2010 samples which displayed different antibacterial profiles. The ethyl acetate extract was chosen for further study, as it demonstrated the most promising antibacterial activity from the initial screen and activity was consistent throughout the sampling timeframe. However, although it inhibited three of the indicator organisms, it cannot be considered broad spectrum, as it was not active against any of the Gram-negative organisms tested. Gram-negative bacteria are usually more resistant to antibiotic substances than Gram-positive bacteria due to the unique outer membrane of the Gram-negative bacterial cell wall that acts as a barrier to most commercial antibiotics (Delcour 2009).

Primary and secondary metabolite production in seaweeds is related to growth and development of seaweed, which in

turn is dependent on environmental factors (Cronin and Hay 1996a; Nishihara et al. 2004; Figueiredo et al. 2008). In the present study, antibacterial activity of a crude *U. lactuca* extract was monitored on a monthly basis in order to examine the effect of season on antibacterial activity. This is the first time a seasonal study has been performed on a green seaweed collected from Ireland. In general, activity was the highest in samples collected in autumn/winter compared to those collected in spring/summer. This trend is consistent with previous work which reported the presence of antibacterial activity from an extract of *Amphiroa bowerbankii* (red alga) in late winter but not in summer (Stirk et al. 2007). This could be related to the photo period the seaweeds were exposed to. For example, secondary metabolite (dictyol B acetate and dictyodial) production in *Dictyota ciliolata* was found to decrease when the seaweeds were exposed to increasing light intensities (Cronin and Hay 1996a).

Production of secondary metabolites in plants is also dependent on different growth phases where precursors will be utilized for different functions such as growth,
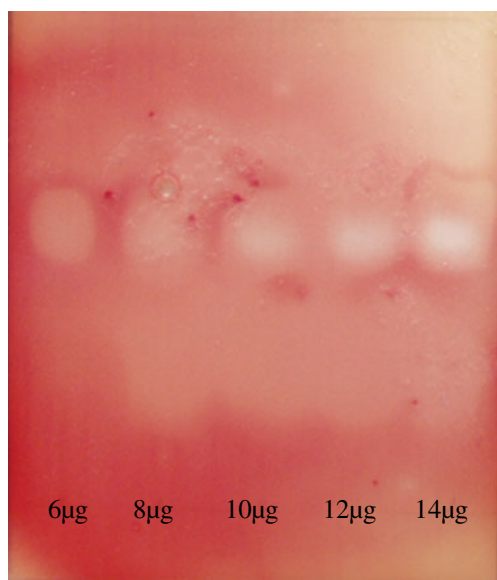

Fig. 5 Dose–response of active sub-fraction SF4 against MR *S. aureus* using bioautography
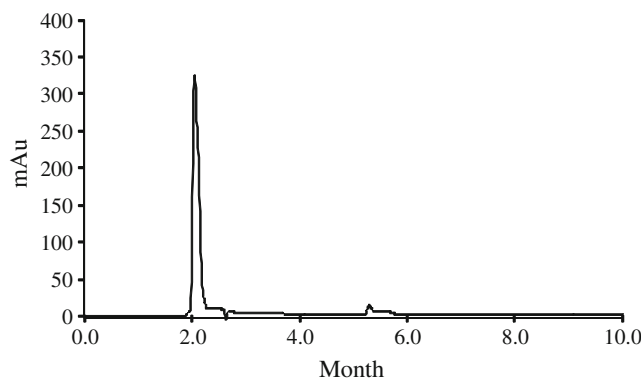

Fig. 6 HPLC analysis of the active sub-fraction (SF4) separated using silica column with a 2:8 THF/hexane mobile phase with detection at 420 nm

differentiation and defence (Cronin and Hay 1996a). The reduced antibacterial activity observed in spring/summer in the present study might be due to fewer precursors being available for production of antibacterial secondary metabolites as compounds are used for the functions outlined above. Antibacterial compounds in seaweeds are also produced in order to protect against microbial attack. For example, younger seaweeds are more susceptible to herbivore grazing and have been reported to produce antibacterial compounds in damaged tissue to combat microbial attack (Hay et al. 1988; Cronin and Hay 1996b).

In summary, production of antibacterial compounds depends on a combination of factors: (1) geographic location; (2) environmental factors such as nutrient availability, temperature and light intensity and (3) physiological factors such as different growth phases and damage from herbivore grazing (Figueiredo et al. 2008). *U. lactuca* analysed in this study consistently produced compounds with activity against *S. aureus*, *B. subtilis* and MR *S. aureus*, indicating that the antibacterial compounds were present throughout the year; however, antibacterial activity varied throughout the study timeframe.

An interesting finding from this study was that a considerable increase in antibacterial activity occurred after the *U. lactuca* extracts were stored for more than 3 months, with activity increasing by 170% after 9 months of storage. This suggests that changes may have occurred to the antibacterial compounds during the storage period which resulted in a more active antibacterial agent. Similar observations were reported previously where an increase in the amount of phenolic compounds was observed in potato peel following frozen storage for more than 4 weeks (Al-Weshahy et al. 2011). However, to date, no other studies have reported increased antibacterial activity from a seaweed sample after prolonged frozen storage under a nitrogen atmosphere. A number of chemical processes are suggested to be responsible for modification of the chemical compounds during prolonged storage, such as acid-catalyzed rearrangements, dimerization/polymerization, isomerization or reactions between alcohol or water with the chemical compounds (Cronin et al. 1995). Further investigation is needed to confirm the exact chemical changes that lead to the increased antibacterial activity observed in the present study.

Bioautography was found to be a more suitable approach than the disc diffusion assay for assessing antibacterial activity in crude *U. lactuca* extracts. Separation of compounds in crude extracts using TLC eliminated the antagonistic effect of inactive compounds. Hence, activity was apparent when bioautography was used but not in the disc diffusion assay. Antagonistic relationships between compounds have been reported previously by Fu et al. (2007) where clove oil alone had a lower MIC against

Aspergillus niger (i.e. was more potent) than when combined with rosemary oil (Fu et al. 2007). However, when the anti-fungal activity was examined using samples containing higher amounts of clove oil than rosemary oil, the MIC value was the same when compared to the activity of clove oil alone. This supports the possibility that there may be similar compounds that act antagonistically with the antibacterial compounds in the ethyl acetate extract of *U. lactuca* in this study. However, once separation of the crude extract is achieved, the disc diffusion assay can then be used to confirm the activity of purified compounds.

As no inhibitory activity was observed for the negative controls in both the disc diffusion and bioautography assays, the activities observed were from the *U. lactuca* extract; however, the mechanisms of action and type of compounds exhibiting the antibacterial activity have yet to be determined. Therefore, separation, purification and identification of the responsible compounds need to be achieved. A combination of preparative TLC separation and column chromatography resulted in a semi-pure antibacterial fraction (SF4). Based on the TLC profile, it was concluded that the antibacterial compounds were within the colourless region that was separated from the other non-active, coloured compounds. These antibacterial compounds were found to be distributed within the F2, F3 and F4 fractions with the greatest activity observed from F2, indicating that the antibacterial compounds were successfully concentrated into this fraction from the simple preparative TLC separation procedure. The presence of antibacterial compounds in the lower fractions (F3 and F4) may have occurred due to the overloading of the preparative TLC plates, resulting in a lag in the migration of the antibacterial compounds. Separation of antibacterial compounds from a crude *Cladophora glomerata* extract was performed using similar techniques and also resulted in relatively pure fractions that were active against fish pathogens (Yuvaraj et al. 2011), indicating that preparative TLC is a valid separation approach.

Although the majority of the antibacterial compounds were collected in SF4, active compounds were also collected in other sub-fractions. However, these may be different compounds with similar structures, as they have different spectra of inhibition. Similar results have been obtained in previous studies where antibacterial compounds of similar structure were active against the same indicator bacteria (Jassbia et al. 2002; Xu et al. 2003; Oh et al. 2008; Chakraborty et al. 2010b). Three diphenolic compounds with similar structures were separated from *Odonthalia corymbifera* by Oh et al. (2008) and were active against *S. aureus*, *B. subtilis*, *Micrococcus luteus* and *E. coli*, but at different activities. However, not all compounds with similar structures would show similar antibacterial activity. For example, the seven labdane diterpenoids isolated from

Ulva fasciata by Chakraborty et al. (2010a) have similar structures, but each of them has its own unique antibacterial profile, which may explain the differences in the antibacterial profiles of SF1, SF7 and SF8 collected from this study. By understanding the molecular structure of these antibacterial compounds, one could examine the mechanism of action, and further modification of these compounds may result in a more potent antibacterial compound(s). For instance, diphenolic compounds with extra bromine isolated from *O. corymbifera* were found to have higher antibacterial activity compared to those with less bromine, indicating that further modification of these compounds resulted in some new synthetic compounds with greater potency (Oh et al. 2008).

The HPLC profile of the purified extract indicated that the fraction was relatively pure, as the antibacterial peak was 97% of the total chromatographic area, and it contained antibacterial compounds that were relatively potent, as 10 μg was sufficient to inhibit the growth of MR *S. aureus*. However, most studies which have successfully isolated pure compounds from macroalgae had higher MIC values, suggesting that the compound(s) isolated in the present study are more potent than those reported previously. For example, pure labdane diterpenoids isolated from the *U. fasciata* by Chakraborty et al. (2010a) had a MIC value of 30 μg against a pathogenic strain of *Vibrio* spp., while a novel bromophenol isolated from *Rhodomela confervoides* had a MIC of 35 μg against *S. aureus* (Xu et al. 2003). Dieckol, an anti-MR *S. aureus* compound isolated from the *Ecklonia stolonifera*, was found to have MIC values ranging from 64 to 128 μg mL^{-1} against different strains of MR *S. aureus* (Lee et al. 2008). These concentrations are far higher than the 10 μg of sub-fraction used in the present study, supporting the hypothesis that the anti-MR *S. aureus* compounds derived from *U. lactuca* are relatively potent and potentially novel.

In conclusion, an ethyl acetate extract obtained from *U. lactuca* contained antibacterial compounds with activity against *S. aureus*, including MR *S. aureus* and *B. subtilis*. The compound(s) was/were active at a low concentration and was/were consistently produced by the seaweed across all seasons, with the highest production observed in the autumn/winter months. Storage of these crude extracts at −20°C under a nitrogen atmosphere for up to 9 months resulted in a considerable increase (170%) in anti-MR *S. aureus* activity. These active compounds were successfully concentrated using a series of separation techniques, including solvent extraction, preparative TLC and column chromatography. This resulted in isolation of a relatively pure sub-fraction with activity against *S. aureus*, *B. subtilis* and MR *S. aureus* at relatively low concentrations. This is the first known report of a successful isolation of a potent anti-MR *S. aureus* compound(s) from *U. lactuca* harvested

in Ireland. Bioautography was found to be the most suitable method for assessing antibacterial activity in crude *U. lactuca* extracts compared to the standard disc diffusion assay. Identification of these potent antibacterial compounds is ongoing. These compounds may have potential applications as alternatives to antibiotics or for use in wound dressings, sterile wipes or hand washes.

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