Antibiofilm and antioxidant activities of extracts of crustose coralline alga *Lithophyllum* sp. from the central Red Sea, Saudi Arabia

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Abstract: In this study, the antibiofilm and antioxidant activities of the crustose coralline alga (CCA) *Lithophyllum* sp. collected from the central Red sea was investigated. The dried CCA samples were extracted with hot water and methanol. Both extracts were tested against marine biofilm forming bacteria to assess the antibiofilm activity. Results showed that methanol extract of the CCA inhibited bacterial growth considerably. The water extract revealed higher antioxidant activity than the methanol extract. The FT-IR spectrum of the CCA showed the presence of functional groups like alkanes, alcohol, carboxylic acid, ethers and esters. In general, results of the present study indicated that CCA produces bioactive compounds, which can inhibit the attachment of unwanted marine bacteria on their surfaces.

Keywords: Crustose coralline algae, coral reefs; chemical defence; biofilm; antifouling; Red Sea

Introduction

Crustose coralline algae (CCA) constitutes an important part in coral reef ecosystems by providing space for the settlement of many organisms including coral species (Webster et al. 2013) and food for herbivores with hardened mouth parts (Steneck, 1997). It has been suggested that CCA makes a substantial contribution to the primary productivity of reef ecosystems due to their abundance (Chisholm, 2003). CCA also act as a cue for the settlement

and metamorphosis of the coral larvae. The efficacy of CCA-associated cues in coral larval settlement and metamorphosis has been elucidated by many previous studies (Hayward and Negri, 1999; Kitamura et al. 2007; Tebben et al. 2015). The settlement mediation effects of CCA may be due to the presence of certain compounds (Morse and Morse, 1991) or associated biofilms (Webster et al. 2004). In addition, CCA is reported to release some chemicals that may serve as antifouling

compounds (Kim et al. 2004; Vermeij et al. 2011).

In the marine environment, both living and non-living surfaces are covered by a microbial layer commonly referred as 'biofilms' (Satheesh et al. 2016). The biofilms associated with marine invertebrates and macroalgae are important for host's defence against the predators or herbivores (Satheesh et al. 2016). Mainly, the microbial communities attached with the marine macroorganisms especially macroalgae are believed to involve in the settlement of invertebrate larval species (Harder et al. 2002; Webster et al. 2004). CCA also provides shelter to a variety of microorganisms and these microbes may play a key role in coral settlement and metamorphosis (Webster et al. 2013).

Most of the marine benthic organisms required to keep their surface free from fouling organisms. Due to this, the organisms express an array of defence strategies starting from mechanical to the production of chemical compounds (Duffy and Hay, 1990; Cronin, 2001; Pansch et al. 2009). There are many previous studies, which reported the settlement-inducing (Pawlik, 1992; Hadfield and Paul, 2001) and inhibiting effects of macroalgal extracts (Walters et al. 2003; Birrell et al. 2008). While CCA is mainly known for its settlement inducing effects on coral larvae (Heyward and Negri, 1999; Raimondi and Morse, 2000; Ritson-Williams et al. 2010; Whalan et al. 2012), their effects against biofilm forming microorganisms is not studied in detail. Hence, in this study, the biofilm inhibiting effects of CCA extracts was

assessed under laboratory conditions against biofilm forming marine bacterial strain. Results obtained in this study will help us to understand the ecological role of CCA in coral reef ecosystems.

Materials and Methods

Crustose coralline algal samples (*Lithophyllum* sp.) were collected from Jeddah coastal waters of the central Red Sea, Saudi Arabia. The samples were kept in sterile polyethylene bags with seawater and transported immediately to the laboratory. In the laboratory, algal samples were washed thoroughly with filtered seawater to remove the epibionts. Nearly 3mm of CCA top layer was separated by bone cutters and powdered using pestle and mortar for solvent extraction. The CCA samples were dried at room temperature under roof shade. The powdered samples were partitioned into two batches, first batch for solvent extraction and the second for hot aqueous extraction. Briefly, 100 g powdered CCA sample of dried plant material was extracted with methanol at room temperature. The extract was filtered through Whatman filter paper (no. 54) and dried by rotary vacuum evaporator. For the hot aqueous extraction, 100 g powdered CCA was extracted in 200 ml of Milli-Q water under 121⁰ C, 15 psi pressure in an autoclave (Tebben et al., 2015). The crude extract was used for the further assay.

Isolation of biofilm-forming bacteria strain for inhibition assays

The biofilm-forming bacteria strain used as target organism for this study was isolated from an artificial substratum (aquaculture net) submerged in the Obhur creek of central Red Sea. The biofilm samples isolated from the substratum was serially diluted and cultured using marine agar. Cultivable bacterial colonies were purified and the dominant strain was isolated for identification. The biofilm-forming bacteria strain was identified tentatively based on biochemical properties as *Idiomarina* sp.

Bacterial growth inhibition assay

Bacterial growth inhibition assay was conducted using the traditional spectrophotometric method. About 2 ml of overnight bacterial culture (1.8×10^8 cell ml⁻¹) was added with 1 mL of the CCA extracts and incubated for 24 h. Two sets of controls, sample control and assay control were maintained for each extract. The sample control was 2ml of sterile broth added with 1 ml of the CCA extracts, whereas the assay control was 2 ml of bacterial culture added with 1 ml of respective solvents used for extraction. The OD (optical density) of the test and control samples was read at 630 nm in UV-Vis spectrophotometer at 1 h interval for 5 h duration.

Biofilm Inhibition Assay-microtitre plate method

The biofilm inhibition assay for all the solvent extracts was determined by the 96-well microtiter plate method described by Coffey and Anderson (2014). An overnight culture of biofilm-forming bacteria strain was adjusted to the OD 1.0 at 645 nm. Bacterial culture of 100

and 50 µl of the solvent extract were added to the sterile 96-well microtiter plate well and the plate was closed and incubated for 24h. The controls were maintained without any extract. After incubation, the plate was inverted to remove the planktonic cells and rinsed with sterile water. After that, 150 µL of 0.1% crystal violet was added to each well of the microtiter plate. After 10 min., the plate was inverted to remove crystal violet stain and again rinsed with sterile water to remove the stains. Finally, 150 µL of glacial acetic acid was added to each well and kept for 10 min and after incubation, the plate was read at 630 nm in a Biotek plate reader.

Total antioxidant activity

The antioxidant activity of the CCA extracts was assessed by the methods described by Prieto et al. (1999). In brief, the extract (0.3 ml) was mixed with 3ml of reagent solution, which consists of 4mM ammonium molybdate, 28mM sodium phosphate and 0.6M Sulphuric acid. The extract along with reagent mixture was taken in a test tube and incubated at 95°C for 90 min. After incubation, the absorbance was measured using a spectrophotometer at 695 nm. The solvents (methanol and water) were used instead of extracts for blank. A calibration curve was prepared using a different concentration of ascorbic acid and total antioxidant activity was expressed as equal to ascorbic acid.

Results and Discussion

The methanol and aqueous extracts of the CCA inhibited the bacterial growth that

was evidenced from the low OD values recorded for the bacterial culture treated with these extracts. Figure 1 explains the growth curve of CCA extracts treated biofilm-forming bacteria strain for a period of 5 h. The OD value was very low for the culture treated with methanol extract. The methanol extract of the CCA showed higher biofilm-inhibitory activity than aqueous extract in microtitre antibiofilm activity (Fig. 2). The bacterial strain without any treatment (control) showed 440% biofilm growth during 24 h period. Bacterial culture treated with aqueous extract exhibited a biofilm growth percentage of 82.27 during this period. However, the methanol extract of the CCA showed negative (-12.18%) biofilm growth (Fig. 2).

Figure 1. Growth curve of biofilm bacteria treated with CCA extracts for 5 h.

Results of this study revealed that CCA extracts could inhibit the settlement of microorganisms on their surfaces. In the marine environment, space is one of the limiting factors, hence most of the organisms from microbes to invertebrates settle on available surfaces if they found that surface suitable. Microorganisms have a tendency to

attach on surfaces and this process on man-made objects submerged in the marine waters attains economical significance. While chemical compounds are mainly used for the control of these biofilms on commercial installations, these chemicals affect the ecosystem health (Satheesh et al., 2016). Natural products from different sources are cited as one of the promising alternatives for antifouling applications and results of this study revealed that bioactive metabolites present in CCA could be used as biofilm inhibitory compounds. Marine algae, in general, possess strong chemical defense against colonizing organisms. The common bioactive metabolites produced by marine macroalgae include polysaccharides, fatty acids, polyphenols and carotenoids (Perez et al., 2016). CCA are reported to have settlement-inducing compounds, which facilitate the settlement of coral larvae (Tebben et al., 2015). While many researchers studied the antifouling activity of CCA and reported that the activity may be due to physical activities such as sloughing (Steneck and Paine, 1986; Puechel et al., 1996; Boas and Figueiredo, 2004). Results of this study showed that CCA also has settlement inhibiting compounds against colonizing marine bacteria.

Figure 2. Inhibitory activity of CCA extracts against biofilm-forming bacteria

The water extract of the CCA showed higher antioxidant activity than the methanol extract (Fig. 3). The CCA extracts also showed concentration dependent antioxidant activity. Generally, cells contain a network of antioxidant defense system, which will scavenge the reactive oxygen species (ROS) (Tejada and Sureda, 2014). Many macroalgal species may produce antioxidants as a defense mechanism against epiphytes. For example, the macroalga *Caulerpa taxifolia* colonized by invasive alga *Lophocladia lallemandii* produce a higher concentration of antioxidant enzymes and other bioactive compounds (Box Centeno et al., 2008). Alternative and safe antioxidant from plant sources attracted wide interest throughout the world (Aliyu et al., 2013). This is due to the emerging evidence on the protective role of plants and vegetables against cancer and other neurodegenerative diseases (Aliyu et al., 2013). The higher antioxidant activity of the CCA extract, particularly water extract indicated that CCA contains antioxidants, which could be used for biotechnological applications.

Figure 3. Antioxidant activity of CCA extracts. The antioxidant activity was expressed as equivalent to ascorbic acid.

The FT-IR spectrum of both water and methanol extract of CCA revealed the presence of the functional groups such as alkanes, alcohol, carboxylic acid, ethers and esters. A C-H stretch between 3000-2840, a CH₂ bending near at 1460 and a CH₃ bending between 1200-1300cm⁻¹ indicate the presence of alkanes. Whereas a broad O-H stretch between 3400-3300 and a C-O stretch between 1260-1000 cm⁻¹ indicate the presence of alcohol. Likewise, an asymmetric C-O-C stretch near 1250 and a symmetric stretch near 1040 cm⁻¹ indicate the presence of aryl alkyl ethers. Similarly, two C-O stretches between 1300-1000cm⁻¹ indicate the presence of esters. Moreover, A C=O between 1730-1700, an O-H broad peak near 3400 and a C-O stretch between 1320-1210 cm⁻¹ indicates the presence of carboxylic acid (Fig 4 & 5).

Figure 4. The FT-IR spectrum of methanol extract of crustose coralline alga *Lithophyllum* sp.

Figure 5. The FT-IR spectrum of water extract of crustose coralline alga *Lithophyllum* sp.

It has been reported that a variety of chemical compounds, which can act against epiphytes/pathogens, are produced by CCA (Kim et al. 2004, Luyen et al. 2009). Therefore, further purification and characterization process could reveal the nature of the compound, which is responsible for bioactivity. Also, the FT-IR result revealed that there are not many changes in between the spectrum of water and methanol extracts, which may be due to the fact that both water and methanol are polar solvents. Because the solubility of the compounds depends on the polarity of the solvents used for extraction (Turkmen et al., 2006; Dai and Mumper, 2010). Therefore, by using non and mid-polar solvents some other novel compounds could also be extracted from CCA. However, considering the results of the antioxidant and antibiofilm activities it is concluded that the CCA produce the compounds, which can inhibit the attachment of unwanted marine

bacteria on their surfaces. Similar antisetlement effects against other invertebrates will be a topic of interest for further studies.

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الأغشية والأنشطة المضادة للأكسدة المستخلصة من الطحالب الحمراء الكلسية لجنس ليثوفيلوم
Lithophyllum sp. من وسط البحر الأحمر، المملكة العربية السعودية

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المملكة العربية السعودية

المستخلص: في هذه الدراسة تم التحقق من الأغشية المضادة والأنشطة المضادة للأكسدة للطحالب الحمراء الكلسية من جنس *Lithophyllum sp.* التي تم جمعها من وسط البحر الأحمر. حيث تم استخلاص عينات الطحالب الحمراء الكلسية المجففة بالماء الساخن والميثانول. ثم تم اختبار كلا المستخلصين الناتجة بالطريقتين السابقتين ضد تكون الأغشية الحيوية البحرية البكتيرية لتقييم أنشطتها المضادة. أظهرت النتائج أن مستخلص الميثانول من الطحالب الحمراء الكلسية يحبط نمو البكتيريا إلى حد كبير، بينما أظهرت عمليات الاستخلاص بالماء أن لها نشاط مضاد للأكسدة أعلى من مستخلص الميثانول. أظهر جهاز طيف الأشعة تحت الحمراء (FT-IR) أن التركيب الطيفي لمستخلص الطحالب الحمراء المتكلسة وجود مجموعات فعالة مثل الألكانات، الكحول، حامض الكربوكسيلك، الاثيرات والاسترات. عموماً أشارت نتائج الدراسة الحالية إلى أن الطحالب الحمراء الكلسية تنتج مركبات حيوية نشطة التي يمكن أن تمنع التصاق البكتيريا البحرية الغير مرغوب فيها على أسطحها.

الكلمات المفتاحية :

الطحالب الحمراء الكلسية ،الشعاب المرجانية ،الدفاع الكيميائي ، الأغشية حيوية ،الأغشية المضادة ، البحر الاحمر .