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Antifouling activity of marine sessile organisms from China against barnacle settlement

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The antifouling activity of a series of hexane, ethyl acetate, ethanol and aqueous extracts from 11 species of marine sessile organisms collected from the south-east coast of China was investigated. Settlement inhibition of cyprid larvae of the barnacle Balanus albicostatus *was used to evaluate their antifouling efficacy. Screening of the 44 extracts showed antifouling activity in 90.9% of the hexane extracts followed by 90.9% of the ethyl acetate, 72.7% of the ethanol and 36.4% of the aqueous extracts. The hexane extracts of* Tubularia mesembryanthemum, Notarcus leachii cirrosus *and* Styela canopus*, the ethyl acetate extracts of* Bugula neritina *and* N. leachii cirrosus*,* and the ethanol extracts of B. neritina *and* Anthopleura sp. *were the most active in inhibiting the settlement of* B. albicostatus*, with* EC_{50} *values all below 50 μg/ml. At least one of the four extracts of each tested species exhibited antifouling activity, suggesting that all 11 marine sessile organisms contained antifouling substances and they may have evolved chemical defences against biofouling on their surfaces.*

Keywords: antifouling, marine sessile organisms, extracts, settlement, barnacle

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INTRODUCTION

Man-made structures in the marine environment, such as ship hulls, cooling systems pipes of power plants and aquaculture equipment, are exposed to surface colonization by marine fouling organisms. This poses serious threats to the safe and efficient operation of these marine structures and constitutes a global technical and economic problem (Richmond & Seed, 1991; Rittschof, 2000; Townsin, 2003; Yebra *et al.*, 2004; Pérez *et al.*, 2006). Although coating surfaces with metal-based antifouling paints, in particular those containing organotins, is the most effective solution to marine biofouling (Alberte *et al.*, 1992; Clare, 1996; Yebra *et al.*, 2004), environmental and human health problems associated with the use of toxic antifoulants (Ellis, 1991; Lau, 1991; Cardwell *et al.*, 1999; Guerin *et al.*, 2007) have led to regulations for, or bans on, their use in a number of countries (Dalley, 1989; Rittschof, 2001; van Wezel & van Wlaardingen, 2004). This makes the development of non-toxic or biodegradable alternatives a necessity (Clare *et al.*, 1992; Fusetani, 2004; Yebra *et al.*, 2004).

Marine sessile organisms are also susceptible to surface biofouling. Epibionts can decrease the fitness or even lead to the death of the host by increasing weight, drag and surface friction; reducing elasticity; interfering with vital processes such as gas exchange, nutrient absorption, excretion and sensing; and enhancing susceptibility to predation (Witman & Suchanek, 1984; Wahl, 1989; Williams & Seed, 1992). Consequently antifouling defence mechanisms are expected to have evolved in some less-fouled marine sessile organisms (Guenther *et al.*, 2007). In fact, many secondary metabolites with antifouling activity are found in many marine invertebrates and seaweeds, suggesting the presence of a chemical defence against epibionts (Clare, 1996; Fusetani, 1998, 2004; Armstrong *et al.*, 2000; Rittschof, 2000, 2001; Steinberg *et al.*, 2001). These naturally occurring antifouling compounds could provide a potential source of environmentally friendly alternatives to toxic antifoulants. Therefore, research on the antifouling activity of secondary metabolites or extracts from such marine sessile organisms could provide important information not only for understanding the chemical mediation of host–epibiont interactions, but also for non-toxic fouling control in marine technology. Thus, in recent years, a large number of studies have focused on screening compounds and extracts from marine organisms for their antifouling activity. In some of these studies, individual compounds are isolated, structurally characterized and tested for their antifouling activity (Okino *et al.*, 1996; Tsukamoto *et al.*, 1997; Cho *et al.*, 2005). On the other hand, crude extracts are also used to evaluate the antifouling activity of marine organisms (Willemsen, 1994; Devi *et al.*, 1998; Cho *et al.*, 2001; Wilsanand *et al.*, 2001; Bhosale *et al.*, 2002; Hellio *et al.*, 2004; Bers *et al.*, 2006). The use of crude extracts is very important and should not be discounted, since it provides a method for the widest possible screening for antifouling substances and because synergism may exist between the different components of an extract.

Therefore, the present study investigated the antifouling potential of crude extracts from various marine sessile organisms. The species chosen all occur abundantly along the

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south-east coast of China and, according to our observations, their body surfaces were less fouled or clean compared to the surrounding organisms. For each species, four extracts were made using solvents of increasing polarity. Antifouling assays were performed against the cyprid larval settlement of the barnacle *Balanus albicostatus* Pilsbry, a notorious fouling macroorganism in the seas of East Asia.

MATERIALS AND METHODS

Collection of marine sessile organisms

Eleven species of marine sessile organisms were collected by hand at several locations along the south-east coast of China (Table 1) during April 2005 to September 2006. The samples were immediately rinsed with freshwater, placed in coolers on ice and transported to the laboratory. These organisms were identified using standard literature and keys with the aid of Professor Shiqiang Zhou, an expert on the taxonomic identification of marine organisms.

Preparation of extracts

Each collected organism was treated in an identical manner. Each species was initially frozen at −20°C and then freeze-dried. The lyophilized material was weighed, ground to powder and sequentially extracted at room temperature with hexane, ethyl acetate, ethanol and distilled water to extract substances with different polarity. To maximize extract collection, dried material was extracted three times with each solvent at an approximately 1:8 (w/v) ratio. After filtration to remove solid fragments, the hexane, ethyl acetate and ethanol extracts were evaporated to dryness using rotary evaporation, and the aqueous extract was lyophilized. All extracts were weighed and stored at −20°C prior to their use. The yield of each extract was determined by dividing the weight of the dry extract by the dry weight of each organism extracted (Table 2).

Table 2. Yield of extracts (% dry weight).

Species	Hexane extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
U. pertusa	0.04	0.18	3.87	22.16
T. hemprichii	0.27	0.25	0.66	9.25
E. acoroides	0.16	0.24	0.79	12.04
T. anhelans	2.07	0.53	1.15	1.68
Halichondria sp.	1.10	0.67	1.82	2.24
B. neritina	0.42	1.42	1.88	17.27
T. mesembryanthemum	0.27	2.36	1.78	2.13
Anthopleura sp.	0.19	3.15	1.50	1.60
N. leachii cirrosus	3.18	3.20	4.42	20.92
S. plicata	0.28	0.50	2.48	8.78
S. canopus	0.11	0.39	0.59	3.56

Full species names are as in Table 1.

Antifouling assay

Antifouling activities of the extracts against barnacle settlement were tested using cyprid larvae of the barnacle *Balanus albicostatus*. Rearing of cyprid larvae was carried out at Xiamen University Marine Biological Laboratory. Adults of *B. albicostatus* were collected together with their rock substrate from intertidal rocks in Xiamen, China. To obtain nauplii for cyprid culture, adults were left to dry overnight and upon immersion in seawater, the nauplius I and nauplius II stages were released. Nauplii were cultured in filtered seawater (FSW; 0.22 μm, salinity 30‰ and temperature 25°C) at a density of 1 larva/ml and fed with the diatom *Chaetoceros muelleri* at a concentration of 2.5×10^5 cells/ml. Each day, nauplii were collected and transferred to fresh algal diet suspensions. After 5–6 days, most of the larvae had metamorphosed to the cyprid stage and cyprids were harvested using filtration. Since it was too difficult to test all the extracts obtained at one time, several batches of cyprids were used.

Settlement assays were conducted in glass Petri dishes (6 cm diameter). The hexane, ethyl acetate, ethanol and aqueous extracts were respectively introduced to the Petri

Table 1. Taxonomy and details of collection of marine sessile organisms.

Species	Location of collection	Zone of collection
Green alga		
Ulva pertusa Kjellman	Tongan (24°38′N 118°11′E)	Intertidal
Seagrasses		
Thalassia hemprichii Ehrenb	Wenchang (19°22′N 110°40′E)	Subtidal
Enhalus acoroides (L.f.) Royle	Wenchang (19°22′N 110°40′E)	Subtidal
Porifera		
Tedania anhelans Lieberkühn	Tongan (24°38′N 118°11′E)	Subtidal
Halichondria sp.	Tongan (24°38′N 118°11′E)	Subtidal
Bryozoa		
Bugula neritina Linnaeus	Zhangzhou (23°56′N 117°30′E)	Subtidal
Coelenterata		
Tubularia mesembryanthemum Allman	Zhangzhou (23°56′N 117°30′E)	Subtidal
Anthopleura sp.	Zhangzhou (23°56′N 117°30′E)	Subtidal
Mollusca		
Notarcus leachii cirrosus Stimpson	Jimei (24°34′N 118°06′E)	Intertidal
Urochordata		
Styela plicata Lesueur	Xiamen (24°26′N 118°04′E)	Subtidal
Styela canopus Savigny	Xiamen (24°26′N 118°04′E)	Subtidal

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dishes using hexane, ethyl acetate, ethanol and FSW respectively as carrier solvent. After evaporation of the organic carrier solvents at room temperature, 30 cyprids were added to each Petri dish containing the extracts in 10 ml FSW or to 10 ml FSW alone as the control. Each extract was tested at concentrations of 0 (control), 10, 50, 100 and 500 μg/ml with cyprids from one batch, and each concentration was assayed in triplicate. All test Petri dishes were incubated at a temperature of 25°C in darkness and examined after 48 hours of incubation. The numbers of settled larvae were enumerated under a stereomicroscope and rates of settled larvae were calculated. Cyprids that became permanently attached and metamorphosed were scored as settled (Rittschof *et al.*, 2003; Hellio *et al.*, 2005).

Statistical analysis

Prior to statistical analysis, percentage settlement values were arcsine-transformed. We used one-way analysis of variance followed by a Dunnett *post-hoc* test for multiple comparisons of treatment means with the control. The significance level was set at $P < 0.05$. The data presented in the figures are not transformed. For each extract, the EC_{50} value (the concentration of extract that reduced the settlement rate by 50% relative to the control) was estimated using the Spearman–Karber method (Hamilton *et al.*, 1977, 1978; Reichelt-Brushett & Michalek-Wagner, 2005). All calculations were based on the test concentrations of the extracts, and the EC_{50} value was given when it was inside the range of concentrations.

RESULTS

In the present investigation, a series of hexane, ethyl acetate, ethanol and aqueous extracts of 11 marine sessile organisms from the south-east coast of China, whose body surfaces are less fouled or clean compared to surrounding organisms in the field, were tested for antifouling activity. The effectiveness of the extracts in inhibiting settlement of *Balanus albicostatus* cyprids is shown in Figure 1 and their EC_{50}, F and *P* values are summarized in Table 3. The 44 extracts were observed to possess various degrees of antifouling activity and, on the basis of their level of activity, the extracts were divided into four groups. Group I comprised the hexane extracts of *Tubularia mesembryanthemum*, *Notarcus leachii cirrosus* and *Styela canopus*, the ethyl acetate extracts of *Bugula neritina* and *N. leachii cirrosus*, and the ethanol extracts of *B. neritina* and *Anthopleura* sp., which were all strongly active in inhibiting barnacle settlement, with EC_{50} values <50 μg/ml. Group II contained extracts exhibiting moderate antifouling activity (50 μg/ml $< EC_{50} < 100$ μg/ml). These extracts were the hexane extracts of *Ulva pertusa* and *Thalassia hemprichii* and *Tedania anhelans*, and the ethanol extract of *Styela plicata*. Group III included extracts showing mild antifouling activity (100 μg/ml $< EC_{50} < 500$ μg/ml) and was the most numerous. Group IV included ethanol and aqueous extracts of *U. pertusa*, *Enhalus acoroides* and *S. canopus*, the aqueous extracts of *T. hemprichii*, *T. mesembryanthemum*, *Anthopleura* sp. and *S. plicata*, the hexane extract of *Anthopleura* sp., and the ethyl acetate extract of *S. canopus*, all of which were inactive in terms of inhibiting the settlement of *B. albicostatus* cyprids at any of the concentrations tested ($P > 0.05$) and with EC_{50} values > 500 μg/ml.

Of the hexane extracts, 90.9% were active, and 9.1% were inactive; of the ethyl acetate extracts, 90.9% were active, and 9.1% were inactive; of the ethanol extracts, 72.7% were active, and 27.3% were inactive; and of the aqueous extracts, 36.4% were active and 63.6% were inactive. It seems that the substances of low or medium polarity, which in the present case were extracted in hexane, ethyl acetate and ethanol, were more likely to be associated with antifouling activity than the high-polarity substances, which were extracted in water. Furthermore, the results highlighted the danger of relying on the extraction of marine organisms using a single solvent for marine natural product antifoulant studies. Clearly, the fact that the extracts from one species of marine organisms showed significantly different activities, varying with the solvent used in the extraction process, suggested that extraction with a single solvent might overlook highly active antifouling compounds, or might even overlook compounds with antifouling activity, thus the wrong conclusions could be drawn.

DISCUSSION

Many secondary metabolites produced by marine organisms are demonstrated to function in defence against consumers, diminishing fouling, inhibiting competitors or microbial pathogens, attracting gametes, and forming the chemical underpinning for many important life processes of marine organisms (Hey & Fenical, 1996). In our work, four crude extracts were prepared for each species tested and at least one extract exhibited antifouling activity, confirming the presence of substances with activity against barnacle settlement in all 11 species. It was suggested that they might all have evolved chemical defences against biofouling on their surfaces. The details for each species are described below.

Large numbers of marine algae possess metabolites with antifouling properties (Bhadury & Wright, 2004). In our study, the hexane and ethyl acetate extracts from *Ulva pertusa* both displayed antifouling activity. However, neither the ethanol nor the aqueous extract showed any inhibition towards the settlement of *B. albicostatus* larvae. This result indicated the low polar nature of the antifouling active component in *U. pertusa*.

Antifouling activity is a poorly investigated property of seagrasses. As far as we know, only three seagrass species, namely *Zostera marina* Linn (Harrison & Chan, 1980; Todd *et al.*, 1993), *Cymodocea rotundata* Ehrenb. & Hempr. ex Aschers. (Bhosale *et al.*, 2002) and *Thalassia testudinum* Banks ex König (Jensen *et al.*, 1998) have been studied for their antifouling activity. Here we explored the antifouling activity of *Thalassia hemprichii* and *Enhalus acoroides* and found that all the crude extracts of *T. hemprichii* other than its aqueous extract, significantly inhibited barnacle settlement ($P < 0.05$), but in the case of *E. acoroides*, only its hexane and ethyl acetate extracts displayed antifouling activity. Furthermore, among the extracts of both *T. hemprichii* and *E. acoroides*, the hexane extract was the most potent in antifouling.

In many studies sponges are a rich source of biogenic compounds with antifouling potential (Sears *et al.*, 1990; Goto *et al.*, 1992; Willemsen, 1994; Clare, 1996). Our results also revealed the presence of antifouling active metabolites in the sponges *Tedania anhelans* and *Halichondria* sp. To our

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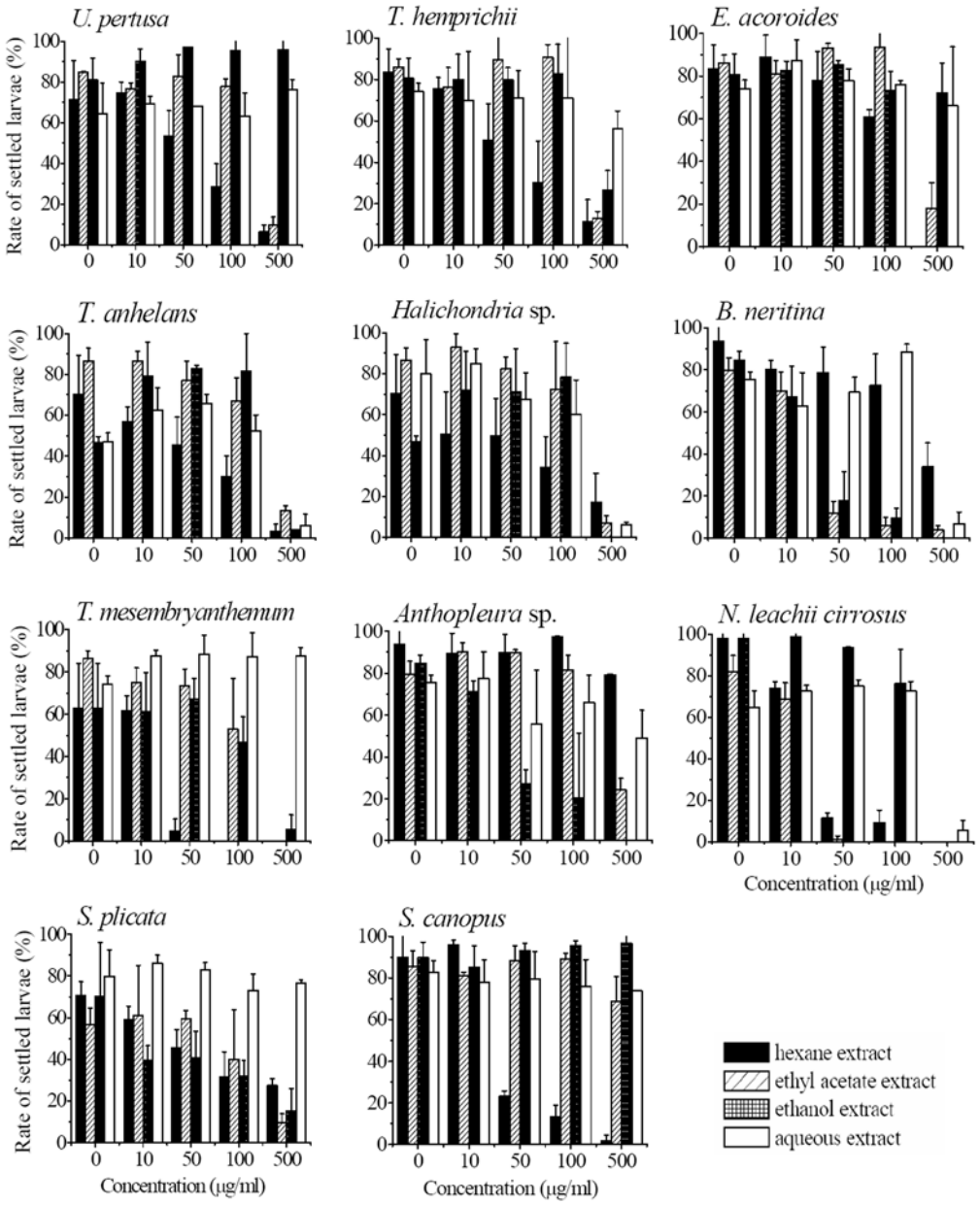

Fig. 1. Effects of hexane, ethyl acetate, ethanol and aqueous extracts from marine sessile organisms on settlement of *Balanus albicostatus* cyprids. Rates of settlement at different concentrations are plotted. Data plotted are the means of three replicates ± standard deviation.

surprise, and different from the other extracts tested in our work, the ethanol extracts of *T. anhelans* and *Halichondria* sp. both significantly induced larval settlement of *B. albicostatus* in the concentration range 10–100 μg/ml. However, at a concentration of 500 μg/ml they exhibited antifouling activity, which was caused by their acute toxicity to *B. albicostatus* cyprid larvae (personal observation). This interesting phenomenon whereby substances with inhibiting activity against barnacle settlement and those with promoting activity for barnacle settlement coexist in one organism is also found in the corals *Leptogorgia virgulata* Lamarck and *Renilla reniformis* Pallas (Standing *et al.*, 1984). This is a logical consequence of the production by sponges and corals of a high number of metabolites with diverse bioactivities and the complexity of the biochemical pathways controlling larval settlement and metamorphosis in the barnacle.

The marine bryozoan *Bugula neritina* is a major target organism for antifouling technology and a settlement inhibition assay with its larvae is often used for screening antifouling active substances (Butler *et al.*, 1996; Perry *et al.*, 2001; Harder *et al.*, 2004; Dobretsov *et al.*, 2007). However, the antifouling activity itself has not received any attention. In our study, we extracted *B. neritina* with four solvents and assayed the extracts for their ability to inhibit the settlement of *B. albicostatus*. As the results show, ethyl acetate and ethanol extracts of *B. neritina* were strongly active in inhibiting barnacle settlement. On the other hand, the hexane and aqueous extracts showed only mild antifouling activity.

When seeking natural product antifoulants from the phylum Coelenterata, most research focuses on corals (Clare, 1996). Other species belonging to the Coelenterata

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Table 3. Antifouling activity of extracts from marine sessile organisms using solvents with different polarity against cyprid larvae of the barnacle *Balanus albicostatus*.

Species	Extract	EC_{50} (μg/ml)	F value	*P* value
U. pertusa	Hexane	88.74 (87.10–90.40)[a]	19.763	0.000
	Ethyl acetate	231.16 (229.18–233.15)[a]	111.342	0.000
	Ethanol	>500[c]	2.530	0.107
	Water	>500[c]	0.987	0.458
T. hemprichii	Hexane	70.10 (68.48–71.75)[a]	9.451	0.002
	Ethyl acetate	256.56 (254.48–258.66)[a]	65.900	0.000
	Ethanol	275.18[b]	14.641	0.001
	Water	>500[c]	0.429	0.784
E. acoroides	Hexane	143.25 (141.48–145.05)[a]	171.488	0.000
	Ethyl acetate	258.28[b]	30.169	0.000
	Ethanol	>500[c]	1.301	0.334
	Water	>500[c]	0.902	0.498
T. anhelans	Hexane	72.99 (71.09–74.94)[a]	20.998	0.000
	Ethyl acetate	202.03 (198.63–205.49)[a]	78.936	0.000
	Ethanol	228.96[b]	36.704	0.000
	Water	224.43 (222.46–226.41)[a]	20.934	0.000
Halichondria sp.	Hexane	111.58 (107.12–116.23)[a]	4.124	0.032
	Ethyl acetate	191.18 (188.86–193.54)[a]	48.471	0.000
	Ethanol	223.61[b]	31.772	0.000
	Water	155.40 (152.93–157.90)[a]	25.231	0.000
B. neritina	Hexane	291.29 (283.47–299.32)[a]	14.000	0.000
	Ethyl acetate	23.21 (22.93–23.49)[a]	86.642	0.000
	Ethanol	22.42 (22.06–22.78)[a]	40.540	0.000
	Water	237.41 (235.93–238.90)[a]	48.899	0.000
T. mesembryanthemum	Hexane	23.64 (23.48–23.79)[a]	53.332	0.000
	Ethyl acetate	131.02 (128.45–133.65)[a]	58.056	0.000
	Ethanol	177.27 (174.86–179.72)[a]	13.758	0.001
	Water	>500[c]	2.086	0.165
Anthopleura sp.	Hexane	>500[c]	3.331	0.056
	Ethyl acetate	293.10 (288.72–297.55)[a]	88.756	0.000
	Ethanol	31.76 (31.12–32.42)[a]	11.408	0.001
	Water	>500[c]	2.237	0.145
N. leachii cirrosus	Hexane	18.94 (18.66–19.22)[a]	178.978	0.000
	Ethyl acetate	19.29 (19.12–19.46)[a]	160.178	0.000
	Ethanol	162.39 (160.62–164.17)[a]	437.645	0.000
	Water	230.75[b]	86.194	0.000
S. plicata	Hexane	100.30 (94.21–106.79)[a]	14.013	0.000
	Ethyl acetate	177.12 (173.76–180.55)[a]	8.821	0.003
	Ethanol	76.95 (73.67–80.37)[a]	4.779	0.029
	Water	>500[c]	1.386	0.313
S. canopus	Hexane	34.72 (34.25–35.20)[a]	46.643	0.000
	Ethyl acetate	>500[c]	3.284	0.072
	Ethanol	>500[c]	1.386	0.313
	Water	>500[c]	0.228	0.914

[a], EC_{50} and 95% confidence limits are presented for each extract; [b], the 95% confidence limits are not reliable, based on analysis of the data from the antifouling assays using the Spearman–Karber method; [c], the EC_{50} values could not be determined because the rate of settled larvae did not fall below 50% in any tested concentration. Full species names are as in Table 1.

have been poorly investigated for antifouling activity, and no mention either of finding significant antifouling active compounds from them or any understanding of the potential chemical defence against surface fouling are made. In our work, hexane, ethyl acetate and ethanol extracts of *Tubularia mesembryanthemum* all exhibited settlement-inhibiting activity against the barnacle *B. albicostatus*, and of these three extracts, hexane displayed the highest level of antifouling activity. Furthermore, barnacle larval settlement was also inhibited in the presence of ethyl acetate and ethanol extracts of *Anthopleura* sp. These results warrant follow-up studies to search for natural product antifoulants in *T. mesembryanthemum* and *Anthopleura* sp.

The sea hare, *Notarcus leachii cirrosus*, which is endemic to China, contains antineoplastic active compounds (Lin *et al.*, 2001, 2002). In our work, the four extracts of *N. leachii cirrosus* were all observed to be antifouling active against the barnacle. In particular, its hexane and ethyl acetate extracts were most potent among all the extracts tested, indicating that *N. leachii cirrosus* also harbours natural products with high antifouling activity and these are lipophilic substances.

Two ascidians of the genus *Styela*, *Styela plicata* and *Styela canopus*, both showed antifouling activity against the barnacle. Although the aqueous extract of *S. plicata* did not exhibit any settlement-inhibiting activity, mild antifouling activity was observed in the hexane and ethyl acetate extracts, and its

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ethanol extract showed moderate antifouling activity. On the other hand, among the *S. canopus* extracts tested, only the hexane extract showed activity and strongly inhibited the settlement of *B. albicostatus*. The present results indicated the very different activities displayed by these two taxonomically close ascidian species. Similar observations are also made by Devi *et al.* (1998) for the soft coral species *Sinularia granosa* Tixier-Durivault, *S. numerosa* Tixier-Durivault, *S. leptoclados* Ehrenberg, *S. minima* Verseveldt, *S. capillosa* Tixier-Durivault, *S. compressa* Tixier-Durivault and *Sinularia* sp., and by Bandurraga & Fenical (1985) for the octocoral species *Muricea californica* Aurivillius and *M. fruticosa* Verrill.

It can be seen that the type of solvent used to obtain extracts from marine sessile organisms can have a great impact on the antifouling activity. In the present study, hexane, a non-polar solvent, was employed for the extraction of non-polar compounds such as waxes, fats and fixed oils; ethyl acetate, a solvent of medium polarity, for the extraction of alkaloids, aglycones and glycosides; ethanol, a solvent of medium polarity greater than ethyl acetate, for the extraction of glycosides; and water, a solvent of high polarity, for the extraction of sugars, amino acids, and glycosides (Houghton & Raman, 1998). A greater percentage of hexane, ethyl acetate or ethanol extracts showed antifouling activity than did aqueous extracts, indicating that low or medium polar compounds are more likely to be associated with antifouling activity than high polar compounds.

In conclusion, the 11 species of marine sessile organisms tested all possessed antisettlement active substances against cyprid larvae of the barnacle *B. albicostatus*. It is suggested that the production of antifouling active metabolites may be common in those marine benthos with a relatively clean surface. Group I extracts were noteworthy for strongly inhibiting barnacle settlement and thus were confirmed as promising sources of natural product antifoulants that should be explored for the development of novel antifouling technology.

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