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Low barnacle fouling on leaves of the mangrove plant *Sonneratia apetala* and possible anti-barnacle defense strategies

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ABSTRACT: A field survey of barnacle fouling on leaves of 5 co-occurring mangrove trees (*Kandelia candel*, *Bruguiera gymnorrhiza*, *Rhizophora stylosa*, *Sonneratia apetala*, and *Avicennia marina*) was conducted in Tongan Bay, southeastern China. The leaves of *S. apetala* were less frequently and less abundantly fouled by barnacles relative to the other mangrove species, suggesting that *S. apetala* may possess anti-barnacle mechanisms. The surface wettability and flexibility of leaves of the 5 investigated species were measured. Except for the lower surface of *A. marina* leaves, the leaf surfaces of all investigated species exhibited low wettability, with water contact angles of 82° to 94°. The leaf flexibility of *S. apetala* was similar to that of *A. marina*, and both were much greater than those of the other 3 species. Leaves of all 5 mangrove species were also subjected to chemical extraction. The extracts of *K. candel*, *S. apetala*, *A. marina*, and *B. gymnorrhiza* showed anti-settlement activities against the barnacle *Balanus albicostatus*, with median effective concentration (EC_{50}) values respectively of 0.85, 10.13, 37.03, and 50.67 µg cm^{-2}. An antifouling compound, identified as oleanolic acid, was isolated from the 2 most active extracts (from *K. candel* and *S. apetala*) via bioassay-guided fractionation. Based on our results, we propose a potential multiple defense strategy of *S. apetala* against barnacle fouling, including low surface wettability, use of an anti-barnacle settlement metabolite of oleanolic acid, and post-settlement detachment of barnacles from its flexible leaves.

KEY WORDS: Mangrove · Barnacle fouling · Antifouling defense · *Sonneratia apetala* · Surface wettability · Oleanolic acid

INTRODUCTION

In the marine environment, surfaces to colonize are often a limited resource, and all natural and man-made surfaces are exposed to the threat of surface fouling by sessile organisms. For living surfaces, the association between epibionts (organisms growing attached to animate surfaces) and basibionts (substrate organisms) is termed epibiosis (Wahl 1989, 2009, Harder 2009) and is among the closest known interspecific associations. The ecological consequences of marine epibiosis can be positive and negative for the organisms involved (Harder 2009, Wahl 2009). Epibiont fouling can cause beneficial effects to the basibiont, such as camouflage and nutrient flow from the epibiont (Feifarek 1987, Thevanathan et al. 2000). However, epibiosis more often appears to be disadvantageous for basibionts. Epibionts can increase weight, drag, and surface friction of the host; cause mechanical and chemical damage to the host; compete with the host for nutrients; interfere with vital processes such as gas exchange, excretion, and sensing; and obstruct host feeding (Wahl 1989, 2009, Bers & Wahl 2004, Harder 2009). Furthermore,

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epibionts can also reduce the amount of light reaching host plants and consequently decrease biomass production (Buschmann & Gómez 1993).

Although surface fouling is common in benthic marine environments, many sessile marine organisms remain relatively clean. This suggests that they have evolved defense mechanisms against epibiosis. Defense strategies include mechanical, physical, and chemical defenses. For instance, removal of macroepibionts can be mechanically achieved by the picking activity of avicularia and vibracularia in bryozoans (Dyrynda 1986). Research on physical defense mechanisms against epibiosis has focused on surface wettability (or surface free energy) and microtopography of body surfaces of marine organisms. Surface wettability can affect settlement of fouling organisms such as barnacles (Dahlström et al. 2004), bryozoans (Rittschof & Costlow 1989), mussels (Carl et al. 2012), and algae (Finlay et al. 2002). Vrolijk et al. (1990) suggested that low surface energy (low surface wettability) may play a role as a natural antifouling defense in the gorgonians *Pseudopterogorgia americana* and *P. acerosa*. Surface microtopography has also been suggested as potentially influencing the settlement of fouling organisms (Callow et al. 2002). Homogeneous structured surface ridges, found on the periostracum of the mussels *Mytilus edulis* (Wahl et al. 1998) and *M. galloprovincialis* (Scardino et al. 2003), have been proposed as a physical antifouling defense for these bivalves.

A widespread adaptation of sessile marine organisms to control epibionts is to produce active antifouling substances. A great deal of research has shown the antifouling efficacy of secondary metabolites from marine invertebrates and seaweeds against potential epibionts. For example, halogenated furanones isolated from the red alga *Delisea pulchra* inhibit the settlement and growth of many ecologically relevant fouling organisms (de Nys et al. 1995, Dworjanyn et al. 2006). To date, a large number of natural products with antifouling activity have been found in a variety of marine organisms including sponges, corals, tunicates, bryozoans, and seaweeds (reviewed by Fusetani 2011, Qian et al. 2015). This suggests that chemical antifouling defenses may be common in sessile marine organisms.

Mangroves, various species of trees that mainly grow in the intertidal zones of tropical and subtropical areas, provide important ecological services, including stabilizing shorelines in coastal streams and estuaries as well as forming habitats for a multitude of species (Nagelkerken et al. 2008). Mangroves are also valuable to humans by supporting coastal fisheries for commercial fish and crustaceans and by supplying a variety of forestry products such as charcoal, firewood, and timber (Walters et al. 2008). In this coastal ecosystem, fouling organisms, particularly common barnacles, are found to attach to the leaves, twigs, and bark of mangrove trees (Ross & Underwood 1997, Sophia Rani et al. 2010). Barnacle fouling creates additional physical drag and weight on mangroves, impedes gaseous exchange, and decreases photosynthesis and food production, which leads to reduced growth, fitness, and survival of mangroves (Santhakumaran & Sawant 1994, Satumanatpan & Keough 1999, Li & Chan 2008, Li et al. 2009). In mangrove plantations created for conservation purposes, barnacle fouling has been suggested as one of the important causes of low seedling survival (Bhat et al. 2004). Given the detrimental effects of barnacle fouling on mangroves, we might expect some species to have evolved defenses against or adaptations to barnacle fouling. Li & Chan (2008) found that mature *Kandelia obovata* and *Aegiceras corniculatum* mangrove trees fouled with barnacles have higher densities of stomata and lenticels, more layers of lower hypodermis, and a thinner palisade layer than non-fouled trees. The authors suggested that these characters could be adaptations to compensate for the impeded gaseous exchange caused by barnacle fouling. Furthermore, a few antifouling secondary metabolites that are active against barnacles have been isolated from the roots of the mangrove species *Ceriops tagal* (Chen et al. 2008, 2011), and extracts of *Avicennia marina* and *Kandelia candel* show anti-settlement effects against barnacles (Deepa et al. 2014), suggesting the possibility of chemical defenses in mangroves. However, information on the defense strategies of mangrove species to reduce barnacle fouling is still very limited.

This study began with an investigation of barnacle fouling on leaves of different mangrove species. We chose leaves for this study because the surfaces of leaves provide an important spatial resource for the settlement and growth of barnacles, and fouling on leaves can cause severe ecological consequences, such as reducing the available photosynthetic surface area and production as well as impeding gas exchange. We quantified the extent of barnacle fouling in the field on leaves of 5 mangrove species (*K. candel*, *Bruguiera gymnorrhiza*, *Rhizophora stylosa*, *Sonneratia apetala*, and *A. marina*). The observation that *S. apetala* exhibited low levels of barnacle fouling on its leaves relative to other co-occurring mangrove species suggests that this species may possess

certain mechanisms to control barnacle fouling on leaves. We also measured physical properties (such as surface wettability and leaf flexibility) of leaves from the 5 mangrove species. Additionally, chemical extracts obtained from these leaves were tested for activity against barnacle settlement and subjected to bioassay-guided fractionation. Based on the results of this study, we propose a multiple defense mechanism of *S. apetala* against barnacle fouling.

MATERIALS AND METHODS

Study site

The study site was situated in an estuary located at Tongan Bay, northeast of the island of Xiamen in southeastern China (24° 38′ N, 118° 11′ E). This site, with a total area of approximately 67 000 m^2, was chosen because of the strong barnacle fouling pressure throughout the year and its inclusion in the mangrove rehabilitation project of the Wetlands and Ecological Engineering Research Centre, Xiamen University. The project was established in response to the nearly complete loss of natural mangroves (with only one wild cluster of small *Avicennia marina* trees remaining) caused by the local development of aquaculture in this area. For this project, the study site was interplanted with mangroves of *Kandelia candel*, *Bruguiera gymnorrhiza*, *Rhizophora stylosa*, and *Sonneratia apetala*. *K. candel* is the dominant species in this study area. Tides are semi-diurnal at this study site.

Barnacle distribution and abundance on leaves of mangroves in the field

Based on their developmental stage, mangrove leaves can be classified into young, mature, and yellow senescent leaves (Kumaresan & Suryanarayanan 2002). In this study, mature leaves of mangrove species were chosen as the study substrata for investigating barnacle fouling because young leaves may be so young that barnacles would not have had sufficient time to settle, and old senescent leaves may soon be shed. The period of mature leaves in the 5 mangrove species investigated here can last for more than 6 mo in the field (Y. Zhang pers. comm.).

The field survey was conducted with the 4 species of planted mangroves (all approximately 1.5 yr old and 80 to 90 cm in height) and a 1.5 m tall cluster of wild *A. marina* trees. *K. candel* was chosen as the control species since it is the dominant species in this study area. Three line transects with lengths in the range of 90 to 100 m were established. In addition to the common *K. candel*, there were moderate densities of *S. apetala*, *B. gymnorrhiza*, or *R. stylosa* around each transect. Each transect was marked every 10 m. At each marked position along the transect, the individual of *S. apetala*, *B. gymnorrhiza*, or *R. stylosa* that was closest to the line transect was investigated for barnacle fouling on its mature leaves, and the nearest *K. candel* individual was selected as the corresponding control in an attempt to ensure that sampling occurred within a homogenous microhabitat. In the case of *A. marina*, the species with only 7 wild trees forming a cluster at this site, we used each of these 7 trees as a replicate. For each replicate, we used the nearest *K. candel* individual as the control. For each *A. marina* tree, branches of the similar height as its *K. candel* control were selected for investigating barnacle fouling on mature leaves.

For each individual mangrove investigated, the frequency with which mature leaves were fouled was determined by counting the number of mature leaves with barnacle(s) attached and comparing it with the total number of mature leaves. To calculate the barnacle density of each plant (each individual mangrove tree investigated was used as a replicate in this study), we counted the total number of barnacles settled on its mature leaves and then divided this value by the total surface area of all mature leaves. To calculate the barnacle density on the upper leaf surface in each plant, we likewise quantified the total number of barnacles on the upper surfaces of mature leaves, and then divided this value by the total area of the upper surfaces of mature leaves. The same method was used for calculating the barnacle density on the lower leaf surface of each plant. To measure the leaf surface area of each plant, both sides of each mature leaf were photographed, with a ruler for scale. These images were analyzed to calculate the leaf surface area using Adobe Photoshop 7.0 software. Barnacles were collected from mangrove leaves and later identified to species based on the morphology of their shell plates.

Physical properties and surface micromorphology of mangrove leaves

Unfouled fresh mature leaves (n = 30) were randomly sampled from each of the mangrove species *K. candel*, *B. gymnorrhiza*, *R. stylosa*, *S. apetala*, and *A. marina* in order to measure leaf area, thickness, and

dry weight of these leaves in the laboratory. The leaf area was measured with image analysis, as described previously. Leaf thickness was measured at the leaf base, middle, and apex using Vernier calipers, and the average of all readings was taken as the thickness of a leaf. The leaves were then dried at 105°C to a constant weight for measurements of the dry weight of leaves.

Flexibility of mangrove leaves was determined using the method of Walters et al. (2003). Fresh mature leaves (n = 90) of each mangrove species were bent around a series of rods with decreasing outer diameter (32.0, 25.5, 18.5, 13.0, 9.5, 7.5, 4.5, 3.0, 2.0, and 1.0 mm) until the leaves broke. All samples broke within this range. The rod diameter at which each sample broke was recorded. Based on these results, the rod diameter at which 90% of the test leaves broke (ED_{90}) was calculated for each mangrove species.

Surface wettability of mangrove leaves was determined by contact angle measurements that were performed as described by Becker et al. (2000). Fresh mature leaves (n = 6) of each mangrove species were randomly sampled to measure the water contact angle using a JC 2000A contact angle goniometer. Drops (1 µl) of double-distilled water were applied to the surfaces of leaves, and the contact angle of each drop on the leaf surface was automatically measured by the contact angle goniometer equipped with computer imaging. For each leaf, contact angles were measured on both upper and lower surfaces.

In this study, surface micromorphology of mangrove leaves was examined via environmental scanning electron microscopy (ESEM). ESEM provides the capability of directly observing fresh biological samples in their natural state. Fresh mature leaves of each mangrove species were cut into small pieces (approximately 5 mm diameter), placed on cylindrical metal slugs using double-sided carbon tape, and then observed with a Philips XL30 ESEM at 1000× magnification. Accelerating voltage was set at 20.0 kV, with water vapor pressure of 5.4 Torr. For each leaf, both upper and lower surfaces were imaged.

Preparation of extracts from mangrove leaves

Mature leaves of the 5 mangrove species investigated here were collected, immediately rinsed with fresh water, and transported to the laboratory where they were frozen at −20°C. The leaves were then freeze-dried and extracted with dichloromethane at room temperature. The extracts were evaporated to dryness under reduced pressure and were then subjected to the barnacle larval settlement bioassay.

Isolation of active compound(s) against barnacle settlement from *S. apetala* leaves

The extract of *S. apetala* was successively fractionated on a silica gel column with 7:3 dichloromethane: ethyl acetate (EtOAc), 4:6 dichloromethane:EtOAc, and 1:9 dichloromethane:EtOAc to provide 12 fractions (F1–F12). Fraction F7 was further chromatographed on a silica gel column and eluted with 10:3 petroleum ether:EtOAc to yield 5 sub-fractions (F7.1–F7.5). F7.4 was subjected to column chromatography using silica gel eluted with 10:4 petroleum ether: EtOAc. Five sub-fractions (F7.4.1–F7.4.5) were obtained. Further purification of F7.4.4 was performed by Sephadex LH-20 column chromatography eluted with methanol to yield 4 fractions (F7.4.4.1–F7.4.4.4), among which F7.4.4.4 was a pure compound. The weight of the compound and the weight of the *S. apetala* extract were measured to calculate the content of the compound in the extract.

Isolation of active compound(s) against barnacle settlement from *K. candel* leaves

The leaf extract of *K. candel* was successively fractionated on a silica gel column eluted with 30:1 petroleum ether:acetone, 10:1 petroleum ether:acetone, and 4:1 petroleum ether:acetone to produce 18 fractions (K1–K18). Fraction K15 was subjected to silica gel column chromatography and elution with 55:1 chloroform:methanol to yield 4 sub-fractions (K15.1–K15.4), among which K15.3 was a pure compound. The weight of this compound and the weight of the *K. candel* extract were also measured.

Structural elucidation of compounds

The structure of compounds was elucidated by comparison of their spectral data (NMR [nuclear magnetic resonance] and ESIMS [electrospray ionization mass spectrometry]) with published values. NMR spectra were obtained in $CDCl_3$ on a Varian Inova 600 MHz NMR spectrometer operating at 600 and 150 MHz for ^{1}H and ^{13}C, respectively, with tetramethylsilane as the internal standard. ESIMS spectral data were measured in positive ion mode on an ABI 3200 Q-Trap mass spectrometer.

Barnacle larval settlement bioassay

The settlement stage of barnacles is the cypris larva, which is specialized for locating a suitable site to attach and metamorphose. Cyprids of the barnacle *Balanus albicostatus* Pilsbry were used to test the anti-settlement activity of the extracts of mangrove leaves, the fractions and pure compounds. The bioassays were carried out as described in detail by Feng et al. (2009). In brief, adult broodstocks of *B. albicostatus* were collected from the intertidal zone in Xiamen, China, and released nauplii when immersed in seawater. Nauplii were reared at 25°C in 0.22 µm filtered seawater (FSW) at a density of 1 larva ml^{-1} and fed with 2.5×10^5 cells ml^{-1} of *Chaetoceros muelleri*. Cyprids were collected after 5 to 6 d and were used in the settlement assays. The experiments were conducted in glass Petri dishes (6 cm in diameter). The extracts, the fractions, and the pure compound were respectively dissolved in dichloromethane. Aliquots of the solution were added to the dishes and spread on the inner surface of the dishes. Control dishes were prepared with only the organic solvent. After complete evaporation of the solvent at room temperature, 10 ml of FSW and 30 cyprids were added to each dish. Three replicates were set up for the control and each of the treatment groups. All test Petri dishes were incubated at 25°C in darkness for 48 h. After this time, the numbers of larvae that settled or died were determined microscopically. Cyprids that permanently attached were counted as settled. Cyprids that did not move and did not respond to being touched by a metal probe with appendages extended were considered dead (Rittschof et al. 1992).

Statistical analysis

Results are generally reported as means ± SE. Statistical analysis was carried out using SPSS version 15.0. Paired *t*-tests were performed to determine the differences in the frequency of barnacle fouling (or the differences in barnacle density) on mature leaves between each of the mangrove species *B. gymnorrhiza*, *R. stylosa*, *S. apetala*, and *A. marina* vs. their corresponding *K. candel* controls. For each mangrove species, differences in barnacle density between the upper leaf surface vs. the lower leaf surface were also analyzed via paired *t*-tests. Differences in barnacle larval settlement between the treatments of the extracts (as well as fractions and pure compound) and control were analyzed by 1-way ANOVA followed by Dunnett's test. The concentration that inhibited settlement by 50% relative to the control (EC_{50}) was calculated using the Spearman–Karber method (Hamilton et al. 1977, 1978, Reichelt-Brushett & Michalek-Wagner 2005). Results were considered significant at $p < 0.05$.

RESULTS

Barnacle fouling on leaves in the field

Field observations revealed that barnacles were the only fouling macroorganisms on leaves of mangroves at our study site. Three barnacle species, viz. *Balanus albicostatus* Pilsbry, *B. amphitrite* Darwin, and *B. reticulatus* Utinomi, were found on leaves of each mangrove species investigated. The number of each barnacle species on leaves was not counted in the field survey, owing to crowding of barnacles which obscured the shell plates and made them difficult to identify.

Among the mangrove species investigated, only leaves of *Sonneratia apetala* exhibited a significantly lower frequency of barnacle fouling and barnacle density compared to leaves of its *Kandelia candel* control (Fig. 1). Only 9.7% of the *S. apetala* leaves were fouled by barnacles, which was 14.2% of the fouling frequency of its *K. candel* control (considered as 100% here), while the frequency of barnacle fouling for other mangrove species was 77.0 to 114.9% of the frequencies of their corresponding *K. candel* controls. In the analysis of fouling frequency, the p-values obtained from paired *t*-tests were respectively 0.020 for the *K. candel*–*Bruguiera gymnorrhiza* pairs, 0.064 for the *K. candel*–*Rhizophora stylosa* pairs, <0.001 for the *K. candel*–*S. apetala* pairs, and 0.442 for the *K. candel*–*Avicennia marina* pairs. The barnacle density on *S. apetala* (0.0097 ind. cm^{-2}) was also the lowest, only 4.1% relative to its *K. candel* control. In contrast, the barnacle density for other mangrove species was 46.8 to 137.1% relative to their corresponding *K. candel* controls. In the analysis of barnacle density, the p-values were respectively 0.272 for the *K. candel*–*B. gymnorrhiza* pairs, 0.044 for the *K. candel*–*R. stylosa* pairs, 0.033 for the *K. candel*–*S. apetala* pairs, and 0.114 for the *K. candel*–*A. marina* pairs.

It is noteworthy that *A. marina* had a significantly greater density of barnacles on the lower leaf surfaces than on the upper leaf surfaces (Fig. 2, $p = 0.018$). No significant differences in barnacle density were detected between the upper and lower surfaces

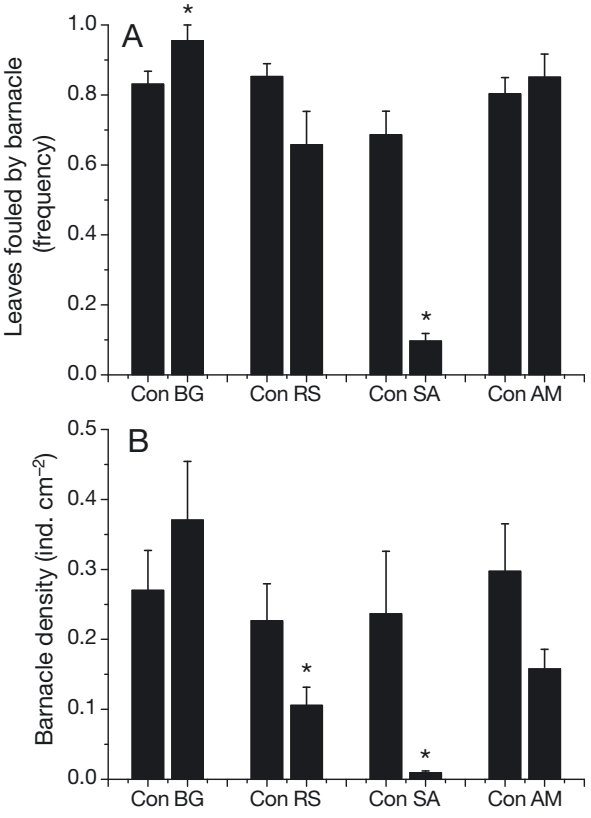


Fig. 1. (A) Frequency with which mature leaves were fouled by barnacles and (B) density of barnacles settling on mature leaves of *Bruguiera gymnorrhiza* (BG), *Rhizophora stylosa* (RS), *Sonneratia apetala* (SA), *Avicennia marina* (AM), and their respective corresponding *Kandelia candel* controls (Con). Data shown are means + SE of replicates (n = 9 for BG, RS, SA, and their corresponding controls, and n = 7 for *A. marina* and its control). Paired *t*-test; *significant difference from control at $p < 0.05$

of leaves in *B. gymnorrhiza* (p = 0.169), *R. stylosa* (p = 0.237), *S. apetala* (p = 0.864), and *K. candel* (p = 0.571; Fig. 2).

Physical properties and surface micromorphology of mangrove leaves

Physical properties of mangrove leaves including leaf area, thickness, weight, flexibility, and surface wettability are presented in Table 1. The mature leaf of *S. apetala* had the lowest leaf area (i.e. the lowest area available for barnacle colonization), the highest thickness value, and the lowest dry weight. Furthermore, the ED_{90} values revealed that the flexibility of *S. apetala* leaves was close to that of *A. marina* leaves, and both were much greater than those of *B. gymnorrhiza*, *R. stylosa*, and *K. candel* leaves.

With the exception of the lower surface of *A. marina* leaves, low surface wettability was found on leaves of all 5 mangrove species, with their mean contact angles being within the narrow range of 82° to 94° (Table 1). On the other hand, the lower surface of *A. marina* leaves exhibited a contact angle of 0° (complete wetting by water). Such a remarkable difference in surface wettability between the lower surface of *A. marina* leaves and the other leaf surfaces seemed closely related to the difference in the surface structure of these leaves (Fig. 3). Only the lower surface of *A. marina* leaves had a dense tomentum of peltate hairs in an irregular shape (water might readily wet the surface through spaces between the hairs), while on all other leaf surfaces, a waxy

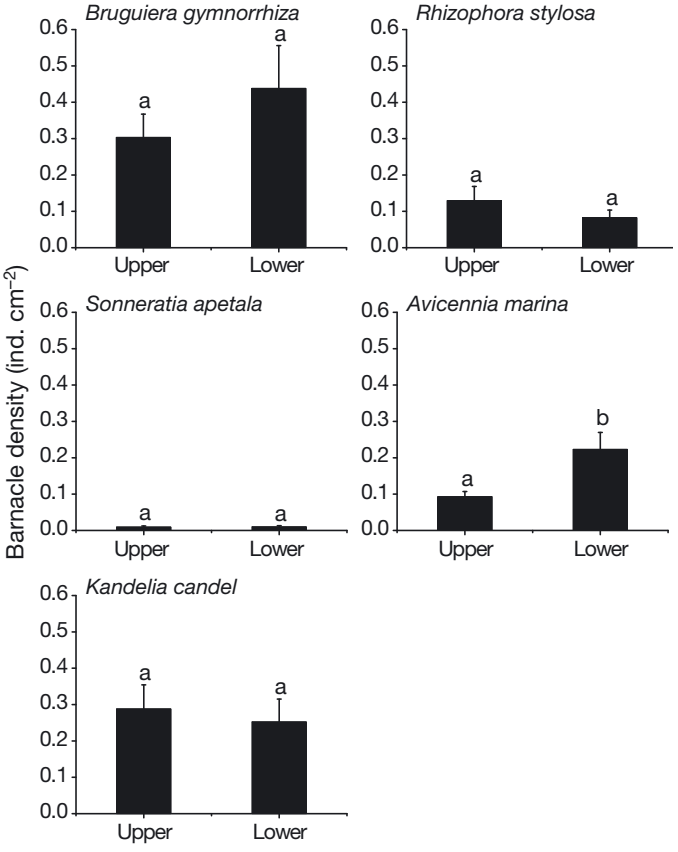


Fig. 2. Differences in barnacle density between upper and lower surfaces of mature leaves of *Bruguiera gymnorrhiza*, *Rhizophora stylosa*, *Sonneratia apetala*, *Avicennia marina*, and *Kandelia candel*. Data shown are means + SE of replicates (n = 9 for *B. gymnorrhiza*, *R. stylosa*, *S. apetala*, and *K. candel*, and n = 7 for *A. marina*). *K. candel* controls used for all species showed no significant difference in barnacle density between the upper and lower leaf surfaces. (*K. candel* data shown here are controls in the investigation of *B. gymnorrhiza*.) Different letters above bars indicate significant differences in barnacle density between upper vs. lower leaf surfaces (paired *t*-test)

Table 1. Physical properties of mature leaves of each investigated mangrove species. Data for leaf area, thickness, dry weight, and water contact angle are shown as means ± SE. Flexibility was measured following Walters et al. (2003) and was quantified by the rod diameter at which 90% of the test leaves broke (ED_{90})

Species	Leaf area (cm^2, n = 30)	Thickness (mm, n = 30)	Dry weight (mg, n = 30)	Flexibility (ED_{90}, mm, n = 90)	Contact angle (°, n = 6) Upper leaf surface	Lower leaf surface
Bruguiera gymnorrhiza	24.77 ± 0.96	1.45 ± 0.07	363.74 ± 66.41	7.59	87.54 ± 1.89	82.21 ± 2.60
Rhizophora stylosa	18.38 ± 1.01	1.01 ± 0.04	381.99 ± 26.18	10.49	91.13 ± 2.11	86.00 ± 1.74
Sonneratia apetala	14.91 ± 0.73	1.61 ± 0.05	168.12 ± 9.46	2.27	82.04 ± 2.06	82.54 ± 1.87
Avicennia marina	18.56 ± 0.69	0.57 ± 0.01	240.77 ± 9.22	1.81	93.04 ± 1.58	0
Kandelia candel	21.55 ± 1.19	0.58 ± 0.01	246.39 ± 14.64	4.86	93.17 ± 1.95	87.58 ± 2.12

(hydrophobic) coating was observed (Fig. 3). Besides the waxy coating, stomata were also found on the lower leaf surfaces of *B. gymnorrhiza*, *R. stylosa*, and *K. candel* as well as on the upper and lower leaf surfaces of *S. apetala*. Salt glands were observed on the upper leaf surface of *A. marina* (Fig. 3). For *A. marina*, the significant difference in barnacle density between the upper and lower leaf surfaces revealed by our field survey may result from their markedly different surface wettability (or surface structure) as described above.

However, surface wettability does not appear to explain the low barnacle fouling on the leaves of *S. apetala* relative to the leaves of other mangrove species. Low surface wettability was found on all of these leaves, with all contact angles close to 90° (except for the lower surface of *A. marina* leaves).

Effects of the extracts from mangrove leaves on barnacle settlement

As shown in Fig. 4 and Table 2, the extracts of *B. gymnorrhiza*, *S. apetala*, *A. marina*, and *K. candel* leaves all exhibited significant inhibitive activities against barnacle settlement ($p < 0.05$), except for the extract of *R. stylosa* leaves, which exhibited no significant difference in settlement between treatments and controls. Based on their EC_{50} values (Table 2), the *K. candel* extract was most active in inhibiting settlement of *B. albicostatus* cyprids, followed by the activity of the *S. apetala* extract. The extract of *S. apetala*, the species that showed significant anti-barnacle capability, and the extract of *K. candel*, the reference species, were subjected to further purification to explore the active compound(s) which may play a role in their high activities.

Table 2. Concentrations of extracts of mature leaves of 5 mangrove species that inhibited settlement of *Balanus albicostatus* cyprids by 50% relative to the control (EC_{50}). Data are expressed as EC_{50} values and 95% confidence limits

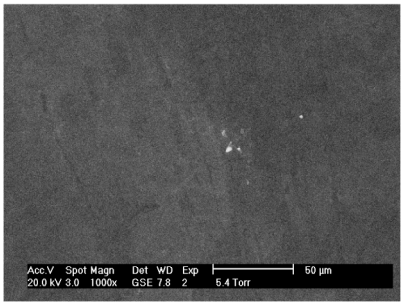
Species	EC_{50} (μg cm^{-2})
Bruguiera gymnorrhiza	50.67 (50.37–50.98)
Rhizophora stylosa	>100
Sonneratia apetala	10.13 (10.00–10.26)
Avicennia marina	37.03 (36.20–37.88)
Kandelia candel	0.85 (0.84–0.87)

Active antifouling compound from mangrove leaves

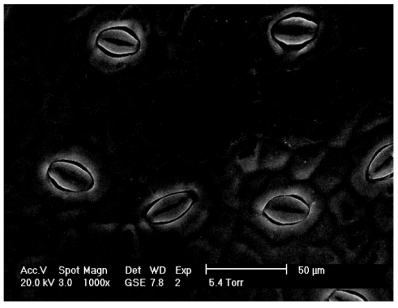
Bioassay-guided fractionation was used during the purification of the extracts of *S. apetala* and *K. candel* leaves (Figs. 5 & 6). One pure active antifouling metabolite was isolated from the *S. apetala* extract. This compound was obtained as a white powder with ESIMS m/z 457 $[M + H]^+$. Based on the ^1H- and ^{13}C-NMR spectral data for the compound and comparisons to those available in the literature (Kizu et al. 1995, Seebacher et al. 2003, Dai et al. 2006), the compound was identified as oleanolic acid (Fig. 7). The content of oleanolic acid in the *S. apetala* extract was 2.04%.

From the extract of *K. candel*, we also isolated 1 pure active compound (Fig. 6). On the basis of its ^1H- and ^{13}C-NMR spectral data, this compound was also identified as oleanolic acid. The content of this compound in the *K. candel* extract was 5.15%.

Oleanolic acid displayed a significant anti-settlement effect against *B. albicostatus* cyprids ($p < 0.05$, Fig. 8), with an EC_{50} as low as 0.06 ± 0.01 μg cm^{-2}. This high inhibitory activity of oleanolic acid against barnacle settlement suggests that this metabolite may play a role in the chemical defense of mangrove leaves against barnacle fouling. This is the first report of antifouling activity of oleanolic acid. Furthermore, it was observed that within the concentra-



BG-upper



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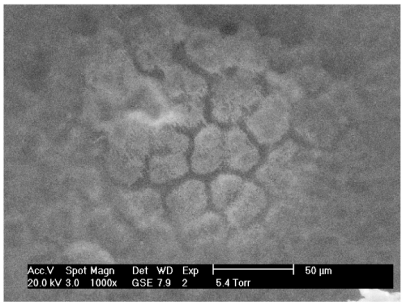

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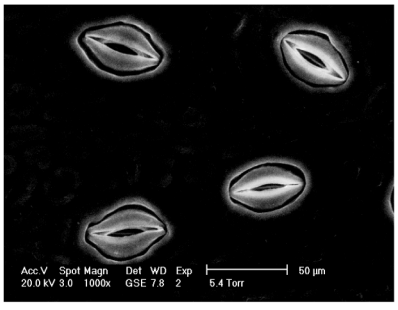

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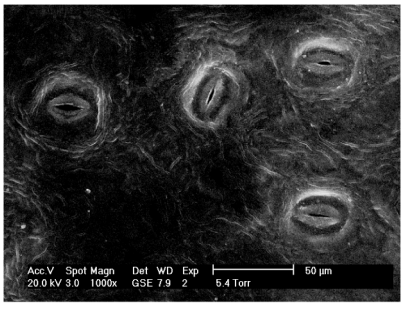

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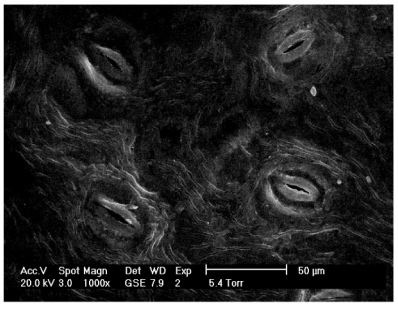

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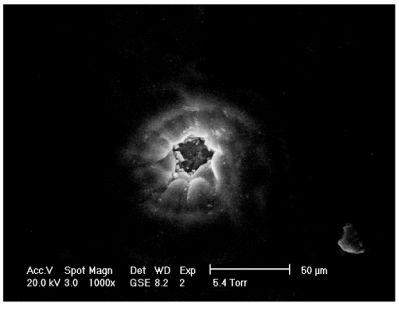

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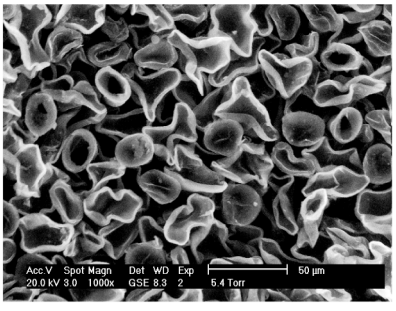

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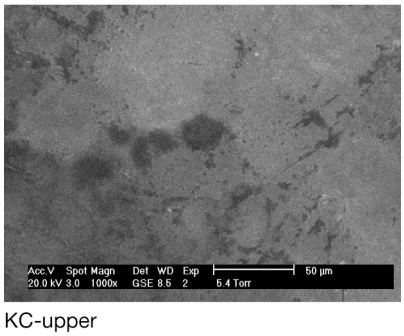
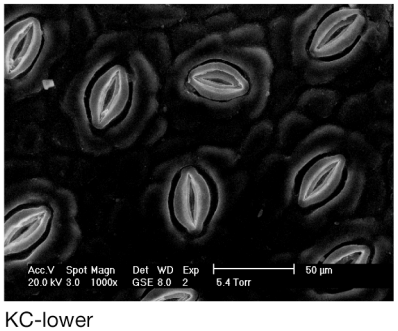


KC-upper



KC-lower

Fig. 3. Environmental scanning electron micrographs of the upper and lower surfaces of mature leaves of each investigated mangrove species. BG: *Bruguiera gymnorrhiza*; RS: *Rhizophora stylosa*; SA: *Sonneratia apetala*; AM: *Avicennia marina*; KC: *Kandelia candel*. Scale bar = 50 µm

tion range of oleanolic acid tested here, there was no significant difference in cyprid mortality in the treatments compared to the control (the lowest p-value was 0.081 when comparing the treatment of 1 µg cm^{-2} and the control). This indicates that this compound inhibited barnacle settlement via a non-toxic mechanism.

DISCUSSION

Barnacles are common fouling species in mangrove ecosystems. Given the ubiquity of barnacles in mangrove forests and the negative consequences of barnacle fouling on mangroves, it seems reasonable to hypothesize that some mangrove species have evolved defense strategies to combat barnacle fouling. Although there is a significant amount of literature on the ecology of barnacles in mangrove forests (e.g. Ross & Underwood 1997, Ross 2001, Satumanatpan & Keough 2001), investigations that rigorously compare barnacle fouling on several different mangrove species in the same habitat are rare, and very little is known about the anti-barnacle defenses of mangrove trees. To our knowledge, the study by Sophia Rani et al. (2010) is the only previous report showing different degrees of barnacle fouling on different co-occurring mangrove species. They found that in the Vellar estuary of India, the barnacle *Balanus amphitrite* exhibited distinct settlement preference for *Rhizophora apiculata* stem surfaces to those of

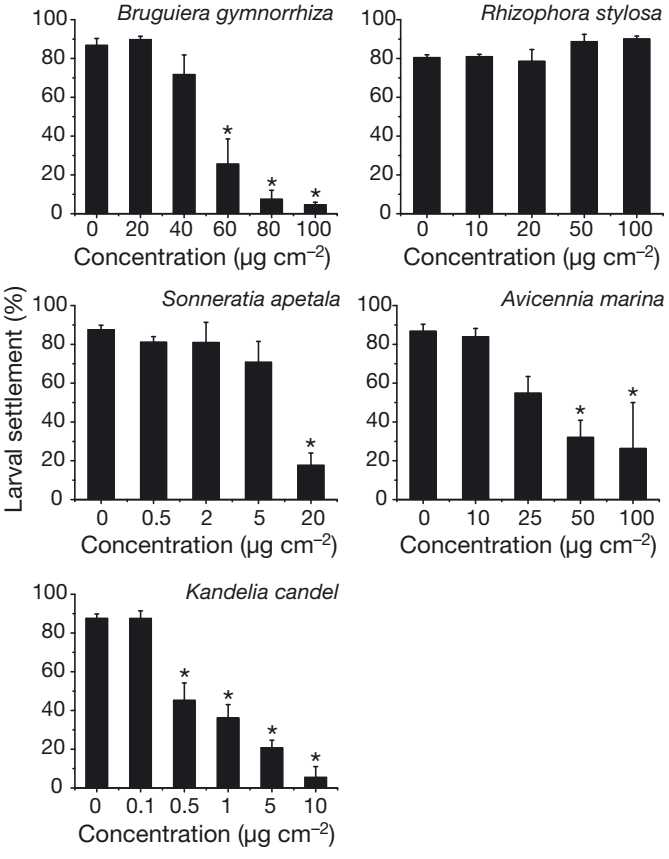


Fig. 4. Effects of the extracts of mature leaves from 5 mangrove species on settlement of *Balanus albicostatus* cyprids. Data shown are means + SE of 3 replicates. One-way ANOVA followed by Dunnett's test; *significant difference from control at $p < 0.05$

Avicennia marina, and they postulated that the different texture of stem bark in these 2 mangrove species might play a role in the different degree of barnacle fouling. In the present work, we found that the leaves of *Sonneratia apetala* at our field site were less frequently and less heavily fouled than those of the other 4 co-occurring mangrove species (*Kandelia candel*, *Bruguiera gymnorrhiza*, *R. stylosa*, and *A. marina*). This indicates the presence of an underlying protection against barnacles in *S. apetala*.

Surface wettability is considered an important physical property that can influence the settlement and adhesive strength of biofouling organisms (Rittschof & Costlow 1989, Dahlström et al. 2004, Finlay et al. 2010). We found low surface wettability of the leaves of all 5 mangrove species investigated, in which the epicuticular wax layer (Fig. 3) of leaves is thought to play an important role (Holloway 1969). Thus, the low surface wettability is unlikely to be responsible for the conspicuously reduced barnacle fouling on the leaves of *S. apetala* compared to the leaves of the other 4 mangrove species. Nonetheless, low surface wettability cannot be excluded as a contributor to the defense against barnacle fouling in leaves of mangroves, as indicated by the case of *A. marina*. In *A. marina* leaves, there was significantly less barnacle density on the upper hydrophobic surface than on the lower hydrophilic surface. This should not be due to the orientation (upper or lower) of the leaf surface, since no significant difference in barnacle density between the upper and lower leaf surface was found in any of the other 4 mangrove plants. Furthermore, in the field, the barnacles seemed to settle much more firmly on the lower leaf surface of *A. marina* than on the other leaf surfaces investigated here (pers. obs.), suggesting that low surface wettability might help decrease the adhesive strength of barnacles on mangrove leaves.

ESEM observation of leaf surfaces (Fig. 3) indicated no unusual surface structures on *S. apetala* leaves (which contain wax and stomata). Thus, some

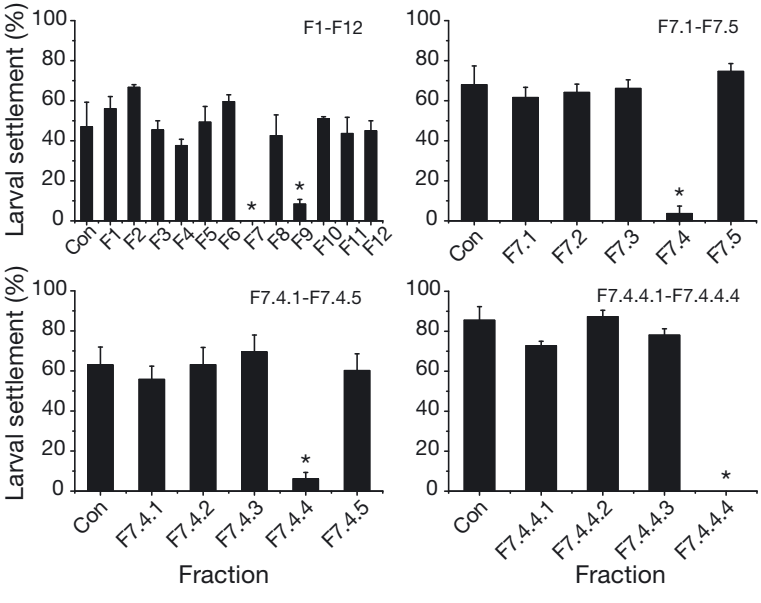


Fig. 5. Effects of the fractions produced during the isolation of oleanolic acid from the extract of *Sonneratia apetala* leaves on settlement of *Balanus albicostatus* cyprids. The concentration for each fraction was 1 μg cm^{-2}. Details for obtaining these fractions are presented in the 'Materials and methods'. Data shown are means + SE of 3 replicates. One-way ANOVA followed by Dunnett's test; *significant difference from control at $p < 0.05$

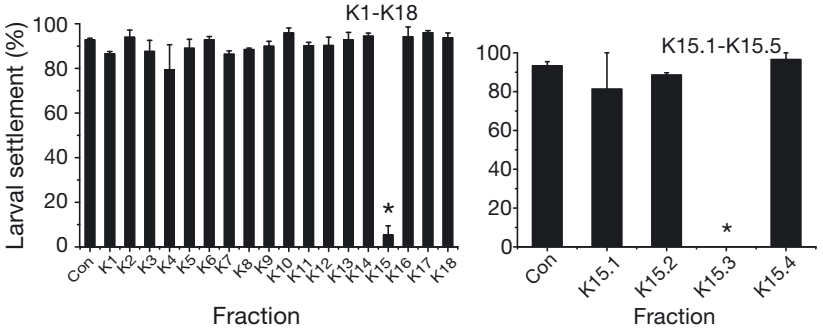


Fig. 6. Effects of the fractions produced during the isolation of oleanolic acid from the extract of *Kandelia candel* leaves on settlement of *Balanus albicostatus* cyprids. The concentration for each fraction was 1 µg cm^{-2}. Details for obtaining these fractions are presented in the 'Materials and methods'. Data shown are means + SE of 3 replicates. One-way ANOVA followed by Dunnett's test; *significant difference from control at $p < 0.05$

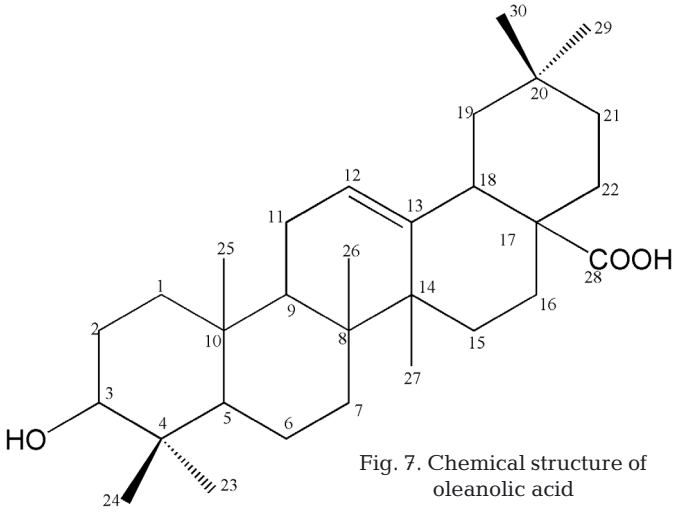


Fig. 7. Chemical structure of oleanolic acid

other anti-barnacle defense(s) are likely involved in *S. apetala* leaves to account for their low barnacle fouling.

The results of the bioassays showed that the extracts of *B. gymnorrhiza*, *S. apetala*, *A. marina*, and *K. candel* leaves were all active in inhibiting settlement of *B. albicostatus* cyprids. The extracts of *A. marina* and *K. candel* were also found to show anti-settlement effects against the barnacle species *B. amphitrite* (Deepa et al. 2014). However, it remains to be demonstrated that barnacle larvae would actually encounter the active substance(s) contained in these extracts under natural circumstances. At present, caution should be observed when suggesting the presence of chemical defenses against barnacle settlement in these mangrove species.

Nevertheless, our results indicate that the possibility of chemical antifouling defense in these mangrove species should not be excluded. Further purification of the *S. apetala* and *K. candel* extracts both produced the same highly active compound, which was identified as oleanolic acid. This compound was also found in leaves of some other mangrove species, including *B. gymnorrhiza* (Ghosh et al. 1985, Ponglimanont & Thongdeeying 2005). Oleanolic acid is a naturally derived pentacyclic triterpenoid compound which exists widely in various plants (Pollier & Goossens 2012). This natural product has been found to exhibit several biological activities, such as hepatoprotective effects as well as antiinflammatory, anti-hyperlipidemic, antibacterial, antioxidant, or antitumor activities (Liu 1995, Pollier & Goossens 2012). Herein, we discovered antifouling activity of oleanolic acid. The results of this study indicate that oleanolic acid inhibited barnacle settlement via a non-toxic mechanism. It should be noted that since this compound was tested on whole barnacle larvae, it is unknown whether oleanolic acid influenced settlement specifically by acting on the pathways controlling larval settlement. Pawlik (1990) suggested that assays testing bioactive compounds by using whole larvae of marine invertebrates are non-specific. Further work is needed to explore the mechanism of action of oleanolic acid against barnacle settlement. Regarding the ecological function of this compound, oleanolic acid and its glycosides may play roles in defense against herbivory and pathogens or in allelopathy in some terrestrial plants (Kubo & Matsumoto 1984, Szakiel et al. 2005, Pollier & Goossens 2012). For example, Kubo & Matsumoto (1984)

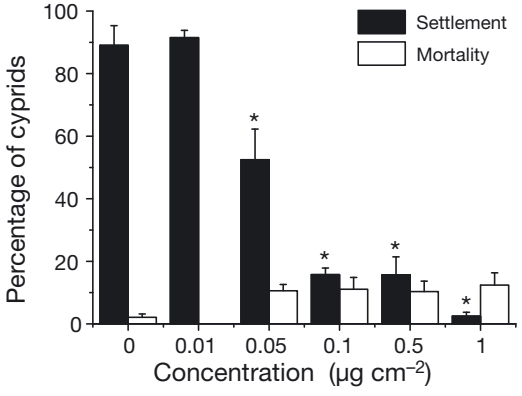


Fig. 8. Effect of oleanolic acid on settlement and mortality of *Balanus albicostatus* cyprids. Data shown are means + SE of 3 replicates. One-way ANOVA followed by Dunnett's test; * significant difference from control at $p < 0.05$

reported that the leaf of the bitter olive *Olea europaea* is coated by almost pure oleanolic acid that helps defend against fungal attacks. Our study suggests a possible antifouling role of oleanolic acid in mangrove species. To confirm that this compound truly functions as an inhibitor of barnacle settlement in mangrove leaves, further research is needed to determine whether oleanolic acid is released onto or near the leaf surface at sufficient concentration(s) to inhibit barnacle settlement, as suggested by Pawlik (1992) when discussing the antifouling role of natural chemicals.

For the *S. apetala* extract, oleanolic acid was isolated from its most active fraction F7 (Fig. 5). As shown in Fig. 5, fraction F9 also exhibited antifouling activity. We found that the active component in F9 was a mixture of phthalate esters (data not shown). Phthalate esters are generally regarded as manufactured chemicals and are widely used in various industries (Staples et al. 1997). Organisms can absorb phthalate esters released into the environment during manufacture, use, and disposal of products (Guven et al. 1990, Staples et al. 1997, Gobas et al. 2003). Further analysis of this component was therefore not performed here.

The extract of *K. candel* displayed higher activity in inhibiting barnacle settlement than that of *S. apetala*. The reason for this is unclear. It is reasonable to suggest that the higher content of oleanolic acid in the *K. candel* extract compared to the *S. apetala* extract is partly responsible. Furthermore, antagonism may exist among the multiple components of the *S. apetala* extract. It is possible that oleanolic acid is differentially available on or near the leaf surface between these 2 species under natural conditions. Based on the results here, it seems that the existence of anti-barnacle settlement substance(s) alone could not explain the significantly less barnacle fouling on *S. apetala* leaves compared to that on *K. candel* leaves.

The abundance pattern of barnacle on mangrove leaves was affected not only by larval supply and settlement behavior of barnacles but also by post-settlement processes. It has been suggested that the post-settlement antifouling mechanisms could explain the lack of fouling on some macrophytes in Hawaiian waters, which included plant flexibility as a physical defense (Walters et al. 2003). In the present work, *S. apetala* leaves were found to be very flexible, with a flexibility much greater than that of *B. gymnorrhiza*, *R. stylosa*, and *K. candel* leaves. We observed that the barnacles could be easily pushed off *S. apetala* leaves by hand with their basal and side plates remaining intact. It is possible that in the field the flexible leaves of *S. apetala* may bend sufficiently when exposed to tidewater movement to cause the weakly attached barnacles to become detached from *S. apetala* leaves. Our personal observation that large barnacle adults were scarce on *S. apetala* leaves is consistent with the above hypothesis, since the chances of detachment would increase as the size of barnacles increases. The post-settlement detachment of barnacles from surfaces has been previously reported in the seaweed *Fucus vesiculosus* and has been suggested as accounting for the low barnacle fouling on this seaweed (Wikström & Pavia 2004). It should be noted that the low barnacle fouling on *S. apetala* leaves could not be sufficiently explained by leaf flexibility alone, since the flexibility of *S. apetala* leaves was slightly lower than that of *A. marina* leaves, and the latter did not show as significant antifouling efficiency. The reasons for this are unknown but may involve the firm attachment of barnacles on the hairy lower leaf surfaces of *A. marina* and the relatively lower antifouling activity of chemicals in *A. marina*.

In summary, a field survey of barnacle fouling on mangroves revealed lower frequency and abundance of barnacles on the leaves of *S. apetala* compared to those of other co-occurring mangrove plants. We propose that *S. apetala* may possess multiple defense systems against barnacle fouling, which consist of several mechanisms probably including low surface wettability, anti-barnacle settlement metabolite of oleanolic acid, and post-settlement detachment of barnacles from its flexible leaves. These mechanisms may operate in overlapping and/or complementary ways to efficiently defend *S. apetala* leaves against barnacles. Although it did not achieve the highest rank for any individual mechanism, *S. apetala* demonstrated the highest antifouling efficacy among the 5 investigated mangrove species via a combination of these mechanisms. It is possible that some of these mechanisms may not have antifouling as a primary aim but still contribute to the defense (McKenzie & Grigolava 1996). Further work is needed to comprehensively understand the complex defense systems of *S. apetala* leaves against barnacle fouling. The complexity of the antifouling strategies of marine organisms has been suggested in the literature (Wahl 2009, de Nys et al. 2010) and demonstrated in some marine species (e.g. Wahl & Banaigs 1991, McKenzie & Grigolava 1996, Bers et al. 2006a,b). Considering the negative ecological effects associ-

ated with barnacle fouling on mangroves, the effective anti-barnacle defense of *S. apetala* (which was introduced to China in 1985 for restoring degraded mangrove forests) may contribute to its successful adaptation and spread in China (Ren et al. 2009).

Investigations on the antifouling defenses of marine organisms are important, especially for developing non-toxic biomimetic technologies for fouling control on artificial structures. The potential multiple defense mechanisms of *S. apetala* against barnacle fouling may contribute to the development of bio-inspired multifunctional coatings, which incorporate a range of attributes to combat marine biofouling (Callow & Callow 2011). In addition, the natural antifouling compound found here, oleanolic acid, is noteworthy for inhibiting barnacle settlement at non-toxic concentrations, suggesting its potential application as an environmentally friendly antifouling agent. Furthermore, oleanolic acid, which is widely distributed in plants (Pollier & Goossens 2012) and abundantly available in nature, which would be an advantage for the commercial application of this compound as an antifoulant.

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