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Herbal plants as a promising source of natural antifoulants: evidence from barnacle settlement inhibition

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A series comprising hexane, ethyl acetate, ethanol and aqueous extracts from six common Chinese herbs (*Carpesium abrotanoides*, *Melia toosendan*, *Cnidium monnieri*, *Vitex negundo*, *Stemona* sp. and *Sophora flavescens*) was investigated for antifouling (AF) activity against cypris (cyprids) larvae of the barnacle *Balanus albicostatus*. All extracts tested except the aqueous extract from *Stemona* sp. significantly inhibited the settlement of cyprids, the most potent being the ethyl acetate extract of *S. flavescens* (EC_{50} value 2.08 μg ml^{-1}), from which an AF compound, identified as 2′-methoxykurarinone, was isolated using bioassay-guided procedures. Furthermore, the AF activity of this compound was found to be highly reversible and greater than that of the three other natural products from *S. flavescens*, namely matrine, oxymatrine and oxysophocarpine. These compounds have been used commercially in China for their pharmaceutical activities, but their AF activities have not previously been evaluated. Analysis of structure–activity relationships suggested that the N-1 nitrogen atom in matrine plays a crucial role in AF activity. Overall, the present findings indicate that herbal plants are a valuable source of novel AF agents.

Keywords: antifouling activity; herbal plants; extract; natural antifouling compound; barnacle; *Balanus albicostatus*; cyprid settlement

Introduction

Biofouling on man-made structures in the marine environment poses serious threats to the safe and efficient operation of these structures by increasing frictional resistance, decreasing the speed and elevating the fuel consumption of ships, increasing the weight of fishing gear and floats and impeding the inflow of cooling water for power plants, and it is one of the most serious problems facing marine technology (Richmond and Seed 1991; Rittschof 2000; Townsin 2003; Yebra et al. 2004; Pérez et al. 2006; Schultz 2007). Although coatings containing biocides such as tributyltin and copper have been widely used to control biofouling (Yebra et al. 2004), negative environmental impacts of certain antifoulants, notably triorganotins, have led to regulations and bans on their use (van Wezel and van Wlaardingen 2004; Yebra et al. 2004). Consequently, a great deal of research has been focussed on finding new antifouling (AF) agents that are not only effective but also non-toxic and/or biodegradable. It has been observed that one of the most promising sources of such compounds is naturally occurring AF agents. So far, a large number of natural product antifoulants has been isolated and identified, including mainly terpenoids, steroids, fatty acids, amino acids, heterocyclics, acetogenins, alkaloids and polyphenolics, most of which are found in marine organisms (eg Clare 1996; Targett 1997; Rittschof 1999, 2001; Fusetani 2004; Yebra et al. 2004; Fusetani and Clare 2006; Paul et al. 2006; Sjögren et al. 2008). Comparatively little attention has been given to terrestrial plants in the search for natural product antifoulants and only a few AF compounds have been isolated from them (Yamashita et al. 1989; Hyodo et al. 1992; Sawant and Wagh 1994; Sawant et al. 1995; Angarano et al. 2007; Pérez et al. 2007; Zhou et al. 2008). For example, *Quercus dentata* Thunb produces the AF active substance kaempferol coumaroylglucopyranoside, which repels attachment of the blue mussel *Mytilus edulis* (Yamashita et al. 1989).

Terrestrial plants are a rich source of natural bioactive products that exhibit a variety of biological activities, many of which have proved to be of considerable significance to humans. Herbal plants are especially effective in preventing infections, controlling disease or enhancing overall health and are used in traditional herbal medicines for multiple therapeutic remedies, eg Jonas (1997), Mahasneh and El-Oqlah (1999), Lin et al. (2001), Pei (2001), Ali et al. (2008). In view of this, it was hypothesized that potent,

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biologically active herbal plants have the potential to disrupt the complex settlement process of marine fouling organisms.

In this study, six herbs commonly used in Chinese medicine were chosen to test the hypothesis. These herbs were *Carpesium abrotanoides* Linn. (Dicotyledoneae, Compositae), *Melia toosendan* Sieb. et Zucc. (Dicotyledoneae, Meliaceae), *Cnidium monnieri* Linn. Cusson (Dicotyledoneae, Umbelliferae), *Vitex negundo* Linn. (Dicotyledoneae, Verbenaceae), *Stemona* sp. (Monocotyledoneae, Stemonaceae) and *Sophora flavescens* Ait. (Dicotyledoneae, Leguminosae). The reasons for choosing these herbs were because they were all easy to obtain from the market at low prices and could be cultivated in large amounts, which would ensure sustainable production of the isolated active compounds, and that they all have been proven to be repellent to terrestrial invertebrates (Xie et al. 1995; Gao et al. 1999; Chandramu et al. 2003; Kaltenegger et al. 2003; Mao and Henderson 2007; Wang et al. 2007). In the present work, their AF properties were assessed and, subsequently, an active compound was isolated from *S. flavescens* using a bioassay-guided fractionation and purification process. The screening of crude extracts, and the isolation, structure elucidation and AF activity of the active compound isolated from *S. flavescens* are described. Furthermore, three commercial natural products, also from *S. flavescens*, namely matrine, oxymatrine and oxysophocarpine (Figure 1), were evaluated for their AF activity. These three compounds have been investigated intensively because of their high pharmacological activities, such as anti-inflammatory, anti-tumor, antipyretic and hepatoprotective effects (Long et al. 2004; Liu et al. 2006; Ling et al. 2007; Zhang et al. 2008). They have also been found to exhibit strong anti-insect activity (Mao and Henderson 2007). These three compounds are extensively used commercially in China as medicines for the treatment of viral hepatitis, cancer, cardiac diseases and skin diseases and as botanical pesticides. However, no reports are known that have evaluated the effect of these compounds on marine fouling organisms and assessed their potential for commercial development as antifoulants. AF assays were performed to test the efficacy of the extracts on the settlement of cyprids (see Aldred and Clare 2008) of the barnacle *Balanus albicostatus* Pilsbry. This work is the first stage towards the commercial development of novel AF agents from herbal plants.

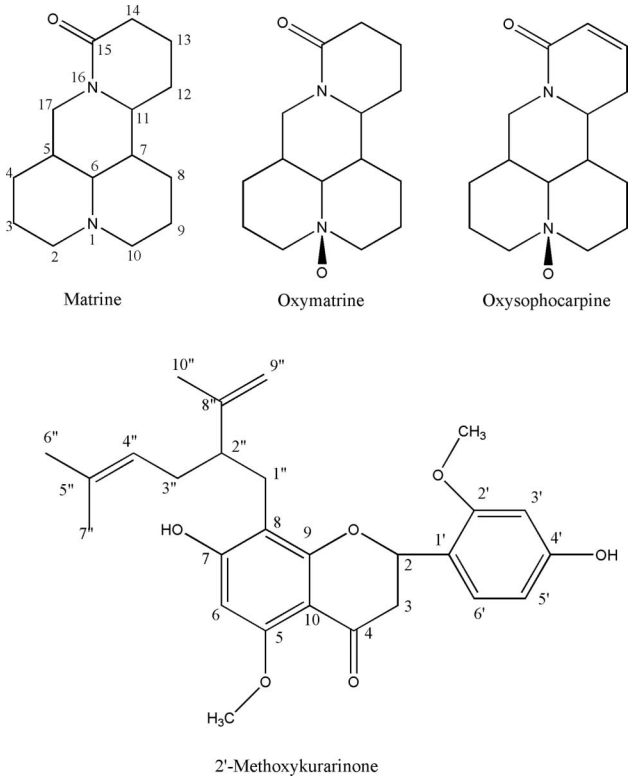

Figure 1. The chemical structures of matrine, oxymatrine, oxysophocarpine and 2′-methoxykurarinone.

Materials and methods

Preparation of plant extracts

The scientific name, local name, life form, distribution and medicinal use of each plant tested are presented in Table 1. The fruits of *C. abrotanoides, M. toosendan, C. monnieri* and *V. negundo*, and the roots of *Stemona* sp. and *S. flavescens* were extracted because these parts of these plants have optimum medicinal efficacy. Dried specimens were obtained from a local herbal medicine store in Xiamen, Fujian Province, PR China and the information provided by the supplier indicated that the fruits of *C. abrotanoides* were collected in Hebei Province, PR China in October 2006; the fruits of *M. toosendan* in Sichuan Province, PR China in July 2006; the fruits of *C. monnieri* and the roots of *Stemona* sp. in Zhejiang Province, PR China during May–June 2006 and the fruits of *V. negundo* and the roots of *S. flavescens* in Hubei Province, PR China during July–August 2006. The species of each plant was authenticated by Professor Z.J. Li, Department of Biology, Xiamen University. The dried tissue samples of each plant species were weighed, ground to powder and extracted at room temperature sequentially with hexane, ethyl acetate (EtOAc), ethanol (EtOH) and distilled water to extract substances with different polarity. Dried tissue (500 g) was extracted three times with each solvent at an ~1:5 (w/v) ratio. After filtration to remove solid fragments, the hexane, EtOAc and EtOH extracts were evaporated under

Table 1. Characteristics of the plants tested.

Scientific name	Local name	Life form	Distribution[a]	Medicinal use[a]
C. abrotanoides	Tian Ming Jing	Herb	Burma, China, Iran, Japan, Korea, Russia, Sikkim, Vietnam	Bronchitis, tonsillitis, boils, ulcers, snakebites and insect bites, round worms, tapeworms, pin worms
M. toosendan	Chuan Lian	Tree	China, Indo-China Peninsula, Japan	Stomach-ache, cholelithiasis, many acute or chronic inflammations, roundworms
C. monnieri	She Chuang	Herb	China, Europe, Korea, North America, Russia, Vietnam	Arthritis, lumbago, edema, headache, common cold, thrombosis, stasis of blood, skin disease, gynecopathy
V. negundo	Huang Jing	Shrub	Bolivia, China, Madagascar, Southeastern Asia	Catarrhal fever, inflammation, toothache, sinuses, scrofulous sores
Stemona sp.	Bai Bu	Herb	Australia, China, India, Indo-China Peninsula, Japan, The Philippines	Respiratory disorders, human and cattle parasites
S. flavescens	Ku Shen	Shrub	China, India, Japan, Korea, Russia	Diarrhea, gastrointestinal hemorrhage, eczema

[a]Information compiled from Editorial Board of Flora of China (1959–2004).

reduced pressure to dryness, and the aqueous extract was lyophilised. All extracts were weighed and stored at -20°C prior to their use.

AF assay

Tests for AF activity were carried out using cyprid larvae of the barnacle *B. albicostatus*. Rearing of cyprid larvae was carried out at Xiamen University Marine Biological Laboratory. Adults of *B. albicostatus* were collected together with their rock substratum from inter-tidal rocks in Xiamen, China. To obtain nauplii for cyprid culture, adults were left to dry overnight, and upon immersion in seawater, broods released the nauplius I and nauplius II stages. Nauplii were cultured in filtered seawater (FSW, 0.22 μm, salinity 30% and temperature 25°C) at a density of 1 larva ml^{-1} and fed with the diatom *Chaetoceros muelleri* at a concentration of 2.5×10^5 cells ml^{-1}. 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 by filtration through a 200 μm mesh size plankton net.

Settlement assays were conducted in glass Petri dishes (6 cm diameter). The hexane, EtOAc, EtOH and aqueous extracts were introduced into the Petri dishes using hexane, EtOAc, EtOH and FSW, respectively, as carrier solvent. After complete evaporation of the organic solvents at room temperature, 10 ml of FSW and 30 cyprids were added to each dish. The extracts were assayed over a concentration range from 0.5 to 1000 μg ml^{-1}. FSW was used as a control because previous pilot studies had shown that there was no significant difference in cyprid settlement between non-treated and organic solvent-treated dishes. Three replicates were set up for the FSW control and for each of the treatment groups. All test Petri dishes were incubated at a temperature of 25°C in darkness and examined after 48 h, at which time the number of larvae that had settled, died or were still swimming was enumerated with the aid of a stereomicroscope. Cyprids that did not move, had extended appendages and did not respond to a touch with a metal probe were counted as dead (Rittschof et al. 1992). Cyprids that were permanently attached and metamorphosed were scored as settled (Rittschof et al. 2003; Hellio et al. 2005). The percentages of settled or dead larvae were analyzed by one-way ANOVA followed by a Dunnett's test for multiple comparisons of treatment means with a control. The significance level was defined as $P < 0.05$. AF activity, which was expressed as the EC_{50} value (the concentration that reduced the settlement rate by 50% relative to the control), and the toxicity, which was expressed as the LC_{50} value

(the concentration that resulted in 50% mortality), were estimated using the Spearman–Karber method (Hamilton et al. 1977, 1978; Reichelt-Brushett and Michalek-Wagner 2005).

Bioassay-guided isolation

As shown in Table 2, of the crude extracts tested in the present work, the EtOAc extract of *S. flavescens* displayed the highest level of AF activity, and so it was chosen for separation of AF compound(s), which were assayed as described above. The dried EtOAc extract (16.6 g) of *S. flavescens* was extracted with methanol (MeOH) and partitioned into MeOH-soluble and -insoluble fractions. AF activity was recognized in the MeOH-soluble fraction (12.7 g), which was subjected to column chromatography using silica gel eluted with chloroform–MeOH (12:1) to give nine major fractions (F1–F9). The active fraction F4 (1.8 g) gave seven sub-fractions (F4.1–F4.7) after silica gel column chromatography and elution with petroleum ether–EtOAc (10:8). Sub-fractions F4.1, F4.2, F4.3 and F4.6 all exhibited AF activity against cyprid settlement at a concentration of 5 μg ml^{-1}, with F4.6 showing the highest activity. Accordingly, F4.6 (140.9 mg) was further chromatographed on a silica gel column and eluted with a petroleum ether–acetone–MeOH (10:5:1) mixture to yield 4 fractions (F4.6.1–F4.6.4). Further purification of the active fraction F4.6.2 (57.7 mg) was performed by silica gel column chromatography using petroleum ether–acetone–MeOH (10:5:1) as an eluting mixture to obtain an active compound (23.6 mg).

Structural elucidation of the active compound

NMR spectra were obtained in $CDCl_3$ on a Varian Unity Plus 500 NMR spectrometer operating at 500 MHz for ^{1}H with tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in parts per million (ppm, δ), and coupling constants (J) are expressed in Hz. ESIMS spectral data were measured in the positive ion mode on an ABI 3200 Q-Trap mass spectrometer. Structural elucidation of the pure compound was based on the interpretation of its spectral data (NMR, MS) and comparison with published values.

Examination of the reversible effect of the compound isolated from S. flavescens

The protocol to determine the possible reversible effect of the compound isolated from *S. flavescens* followed that described in Sjögren et al. (2006). Thirty cyprids

Table 2. Yield, AF activity and toxicity of extracts from six species of Chinese herbal plants.

Species	Crude extract	Yield (% dry weight)	EC_{50} (μg ml^{-1})	LC_{50} (μg ml^{-1})
C. abrotanoides	Hexane	4.48	18.34	>25
	Ethyl acetate	2.95	7.73	21.77
	Ethanol	1.34	10.87	>50
	Water	0.76	242.10	>1000
M. toosendan	Hexane	0.92	96.14	>100
	Ethyl acetate	0.56	7.06	17.50
	Ethanol	4.04	36.90	>50
	Water	2.87	588.71	>1000
C. monnieri	Hexane	7.08	9.44	>50
	Ethyl acetate	7.35	14.04	223.61
	Ethanol	2.98	35.12	>50
	Water	6.05	173.78	>1000
V. negundo	Hexane	0.32	45.55	>50
	Ethyl acetate	0.89	16.55	62.52
	Ethanol	0.45	20.63	>50
	Water	0.43	869.55	>1000
Stemona sp.	Hexane	0.13	6.98	17.86
	Ethyl acetate	0.63	4.98	42.76
	Ethanol	3.35	57.00	>50
	Water	5.32	>1000	>1000
S. flavescens	Hexane	0.71	67.56	>500
	Ethyl acetate	3.32	2.08	8.15
	Ethanol	2.13	29.26	33.36
	Water	6.55	297.88	>1000

(three replicates) were exposed to the compound isolated from *S. flavescens* at the effective concentration of 5 μg ml^{-1} (see the Results section) for 48 h. After 48 h, all unsettled cyprids were counted, washed and transferred to FSW. The dishes were maintained for another 48 h and then investigated for the numbers of settled larvae. Dishes in which cyprids were maintained in the compound isolated from *S. flavescens* at 5 μg ml^{-1} throughout the experiment served as a control.

Evaluation of AF activity of three commercial natural products from S. flavescens

In the present work, the AF activity of three commercial natural products from *S. flavescens* (matrine, oxymatrine and oxysophocarpine) were examined and compared with that of the compound isolated from *S. flavescens* in this study. Matrine, oxymatrine and oxysophocarpine with purity >95% were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Their chemical structures are shown in Figure 1. They were tested using cyprids of *B. albicostatus* as described above.

Results

Crude extract yield and AF activity

To confirm the existence of AF substances in the plant species tested, a suite of increasing hydrophilic extracts (hexane, EtOAc, EtOH and aqueous extracts) was prepared from each plant. The yields of each of the four crude extracts obtained from each plant species are summarized in Table 2. The AF activities of all the extracts and their toxicities against cyprids of *B. albicostatus* expressed as their EC_{50} and LC_{50} values are also shown in Table 2. Comparison of means using ANOVA and Dunnett's test revealed that all extracts, except the aqueous extract from *Stemona* sp., significantly inhibited the settlement compared with the control ($P < 0.05$), suggesting that these six plants contain AF substances. Moreover, most of the extracts tested yielded EC_{50} values much lower than their LC_{50} values, indicating that these extracts have the potential for the isolation of non-toxic or low-toxicity AF compounds from them.

In addition, the extracts were observed to possess various degrees of AF activity. For each species, the organic extracts were much more effective in inhibiting cyprid settlement than the aqueous extract. It seems that the substances of low or medium polarity, which in the present case were extracted in hexane, EtOAc and EtOH, were more likely to be associated with high AF activity than the high-polarity substances, which were extracted in water. In particular, among the 24 extracts tested, 11 extracts, ie the hexane extracts of *C. abrotanoides, C. monnieri* and *Stemona* sp., the EtOAc extracts of *C. abrotanoides, M. toosendan, C. monnieri, V. negundo, Stemona* sp. and *S. flavescens* and the EtOH extracts of *C. abrotanoides* and *V. negundo* exhibited promising levels of activity, with EC_{50} values < 25 μg ml^{-1}, the standard requirement established by the US Navy program as a potency criterion for natural antifoulants (Rittschof 2001). The most promising activity was shown by the EtOAc extract of *S. flavescens*, which inhibited cyprid settlement at an EC_{50} of 2.08 μg ml^{-1}. On the basis of these results, the EtOAc extract of *S. flavescens* was chosen for isolation of the AF active compound.

Isolation and identification of 2′-methoxykurarinone

Using bioassay-guided fractionation, one AF compound was isolated from the most active fraction of the EtOAc extract of *S. flavescens* in the present investigation. This compound was obtained as a pale yellow powder with ESIMS m/z 453.4 $[M + H]^+$ and ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H: 7.38 (1H, d, $J = 8.0$ Hz, H-6′), 6.49 (1H, dd, $J = 8.0$, 2.0 Hz, H-5′), 6.45 (1H, d, $J = 2.0$ Hz, H-3′), 6.10 (1H, s, H-6), 5.63 (1H, dd, $J = 13.5$, 2.5 Hz, H-2), 5.01 (1H, br t, H-4″), 4.72 (1H, br s, H-9″ a), 4.66 (1H, br s, H-9″b), 3.79 (3H, s, 2′-OCH_3), 3.75 (3H, s, 5-OCH_3), 2.85 (1H, dd, $J = 16.0$, 13.5 Hz, H-3α), 2.62 (3H, m, H-1″, H-3β), 2.38 (1H, m, H-2″), 2.05 (2H, m, H-3″), 1.66 (3H, s, H-10″), 1.62 (3H, s, H-7″) and 1.51 (3H, s, H-6″).

In the aromatic region of the ^{1}H NMR spectrum, a singlet appearing at δ6.10 (1H, s, H-6) was assigned to the proton of a tri-substituted ring A. The protons at δ7.38 (1H, d, $J = 8.0$ Hz, H-6′), 6.49 (1H, dd, $J = 8.0$, 2.0 Hz, H-5′), 6.45 (1H, d, $J = 2.0$ Hz, H-3′) indicated the existence of an ABX coupling system, which suggested that the B ring was oxygenated at C-2′ and C-4′. The signals at 3.79 (3H, s, 2′-OCH_3), 3.75 (3H, s, 5-OCH_3) suggested that C-2′ and C-5 were substituted with methoxyl groups. The three proton double doublets at δ 2.85 (1H, dd, $J = 16.0$, 13.5 Hz, H-3α), 2.62 (1H, overlapped, H-3β) and 5.63 (1H, dd, $J = 13.5$, 2.5 Hz, H-2) indicated the presence of a flavanone skeleton. The other signals at 5.01 (1H, br t, H-4″), 4.72 (1H, br s, H-9″a), 4.66 (1H, br s, H-9″b), 2.62 (2H, overlapped, H-1″), 2.38 (1H, m, H-2″), 2.05 (2H, m, H-3″), 1.66 (3H, s, H-10″), 1.62 (3H, s, H-7″), 1.51 (3H, s, H-6″) indicated a lavandulyl group. By comparison of the ^{1}H-NMR and mass spectral data with those in the literature (Kang et al. 2000; Kim et al. 2006), the compound was identified as 2′-methoxykurarinone (Figure 1).

AF activity of 2′-methoxykurarinone and its comparison with three commercial natural products from S. flavescens

Settlement of cyprid larvae of *B. albicostatus* was significantly inhibited in the presence of 2′-methoxykurarinone. The EC_{50} value of 2′-methoxykurarinone was 2.02 μg ml^{-1}, whereas its LC_{50} value exceeded 25 μg ml^{-1} (Table 3), indicating that this compound inhibited larval settlement via a non-toxic mechanism. Furthermore, the AF activity of 2′-methoxykurarinone against barnacle settlement was found to be reversible. After exposure to the effective concentration of this compound for 48 h, the cyprids were transferred to FSW and the ability to complete settlement and metamorphosis was recovered by almost 70% within 48 h (Figure 2), further substantiating the non-toxic nature of the compound.

Figure 3 and Table 3 also represent the results of AF assays of three commercial natural products from *S. flavescens*, namely matrine, oxymatrine and oxysophocarpine. It is noteworthy that only matrine was found to have high AF activity (EC_{50} 7.14 μg ml^{-1}) and weak toxicity (LC_{50} > 250 μg ml^{-1}). Neither oxymatrine nor oxysophocarpine showed any inhibition of barnacle settlement. Overall, among the four natural products from *S. flavescens* tested, 2′-methoxykurarinone, obtained in the present work, was the most active antifoulant.

Discussion

B. albicostatus, the organism chosen for the AF assays, is an acorn barnacle abundant in the inter-tidal areas of Korea, Japan and China (Utinomi 1967; Newman and Ross 1976; Lee and Kim 1991), and is one of the dominant foulers in East Asian waters being frequently found attached to aquacultural nets, buoys and dock pilings (Huang and Cai 1984; Chen 2006). To date, many characteristics of *B. albicostatus*, including larval morphology, development, metamorphosis, growth, phylogeny and reproduction, have been investigated (Iwaki 1981; Lee and Kim 1991; Nakamura 1997; Puspasari et al. 2000, 2001; Khandeparker et al. 2005; Chen 2006; Desai et al. 2006; Chan and Leung 2007). Furthermore, it was convenient to collect *B. albicostatus* as it is reproductive throughout the year in Xiamen, PR China and synchronous mass cultures of cyprid larvae can be easily maintained in controlled laboratory conditions. *B. albicostatus* has also been used previously as a model organism for screening natural AF agents from the mangrove plant *Ceriops tagal* in the authors' laboratory (Chen et al. 2008; Feng et al. 2008).

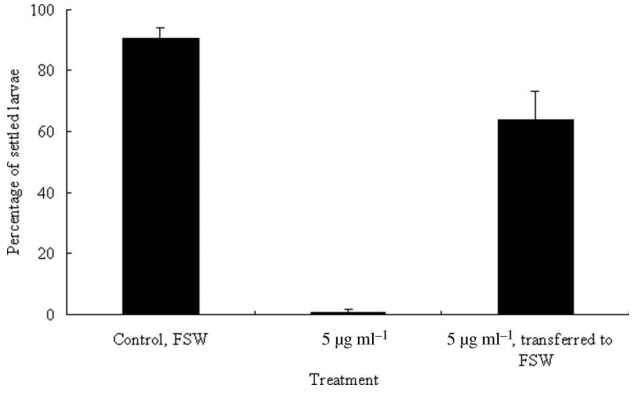

Figure 2. The reversible effect of 2′-methoxykurarinone. Dishes in which cyprids were maintained in 5 μg ml^{-1} 2′-methoxykurarinone throughout the experiment are labelled as 5 μg ml^{-1}. Dishes in which cyprids were exposed to 5 μg ml^{-1} 2′-methoxykurarinone for 48 h before washing and transferring to seawater are labelled as 5 μg ml^{-1}, transferred to FSW. Data shown are means + SD of three replicates.

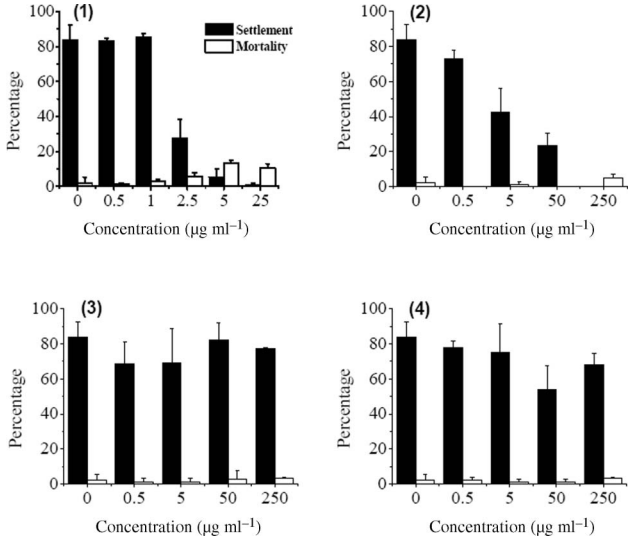

Figure 3. Effects of 2′-methoxykurarinone (1), matrine (2), oxymatrine (3) and oxysophocarpine (4) on settlement and mortality of cyprids of *B. albicostatus*. Data shown are means + SD of three replicates.

Table 3. AF activity and toxicity of four natural products from *S. flavescens* against cyprids of *B. albicostatus*.

Compound	EC_{50} (μg ml^{-1})	LC_{50} (μg ml^{-1})
2′-Methoxykurarinone	2.02	>25
Matrine	7.14	>250
Oxymatrine	>250	>250
Oxysophocarpine	>250	>250

The present investigation focussed on examining the AF activity of six herbal plants against the settlement of cyprids of *B. albicostatus* and isolating natural products with potential as future AF agents. All the plants tested in this study have been proven to be repellent to terrestrial invertebrates and their activities in expelling worms, and insects in particular have received considerable attention (Editorial Board of Flora of China 1959–2004; Xie et al. 1995; Gao et al. 1999; Chandramu et al. 2003; Kaltenegger et al. 2003; Mao and Henderson 2007; Wang et al. 2007). Moreover, there have been many reports concerning their chemical constituents (eg Kitajima et al. 2001; Lee et al. 2002; Kaltenegger et al. 2003; Kim et al. 2006; Sathiamoorthy et al. 2007; Xie et al. 2008). However, there have been no reports concerning the AF activity of their constituents against marine invertebrates.

In this study, the results from the settlement experiments with crude extracts showed that all the herbs tested contained substances that inhibited larval settlement of *B. albicostatus*. Furthermore, for each species tested, of the four crude extracts, at least three extracts exhibited AF activity. It could be inferred that there might be more than one AF active compound in each herb. On the basis of the present findings, it is suggested that the production of AF active metabolites may be common in herbal plants and thus they may have great potential as a source of natural AF compounds. Furthermore, many herbal plants have the advantages of being easily collected and suitable for mass culture. In general, the production of natural product antifoulants on a large scale is one challenge for AF technology, because to date most have been isolated from marine organisms such as rare corals, sponges and other invertebrates that are not available in sufficient quantities to be harvested on a commercial scale (Rittschof 2001). However, many species of herbal plants are distributed widely around the world (Editorial Board of Flora of China 1959–2004) and because of the rapid development of the herbal industry, many have been cultivated on an industrial scale and/or mass culture techniques have been developed (Yang et al. 1999).

Among all the extracts tested, 11 appeared to be of interest because of their high inhibitory activities against barnacle settlement, with EC_{50} values $<25\ \mu g\ ml^{-1}$, indicating the presence of potent AF compounds. In the present work, as the EtOAc extract of *S. flavescens* was found to be the most active against *B. albicostatus*, it was chosen as an example for the isolation of an AF compound. From this extract, one lavandulyl flavanone with high activity, viz. 2′-methoxykurarinone, was isolated. Interestingly, eight flavone and isoflavone derivatives that have been isolated from terrestrial plants and share a similar molecular skeleton with 2′-methoxykurarinone, show strong anti-settlement activities against the barnacle *B. amphitrite* (Zhou et al. 2008). On the other hand, although the bioassay-guided fractionation approach used here identified 2′-methoxykurarinone, it must be remembered that this compound was isolated from the most active F4.6 sub-fraction and there are three other active sub-fractions yet to be purified (see the Materials and methods section). Thus, some other compounds in the EtOAc extract of *S. flavescens* may also have inhibiting action against cyprid settlement. The present results revealed that 2′-methoxykurarinone was one of the compounds responsible for the observed AF activity of the crude extract and it is probably the most active antifoulant. Previous studies have demonstrated that 2′-methoxykurarinone has anti-malarial, glycosidase inhibitory and cytotoxic activities (Kang et al. 2000; Kim et al. 2004, 2006). The present findings are the first laboratory evidence of the strong inhibitory activity of 2′-methoxykurarinone on the settlement of barnacle larvae, along with relatively low toxicity. Furthermore, the AF effect of 2′-methoxykurarinone was shown to be highly reversible. These observations suggest that this compound probably functions by some mechanism other than toxicity and 2′-methoxykurarinone could be used as an environmental-friendly AF component in marine paints.

As a widely used medicinal herb, *S. flavescens* has commanded attention in natural product research and has been found to be rich in quinolizidine alkaloids, flavonoids and triterpenoids (Tang and Eisenbrand 1992). Matrine, oxymatrine and oxysophocarpine, the major quinolizidine alkaloids in *S. flavescens* (Zhang et al. 2008), have been investigated intensively and commercialized as medicines and botanical pesticides in China because of their high pharmacological activities. The present study presents the first evaluation of the AF activities of these three compounds and compares their effects with that of 2′-methoxykurarinone, the active compound isolated from *S. flavescens*. Although oxymatrine and oxysophocarpine did not show any significant effect on the settlement of cyprids, matrine by contrast exhibited high inhibition of settlement, along with the lack of observed toxicity, thereby indicating its potential as a future AF agent. Some useful information about the structure–activity relationship could be obtained by considering the similar structures of these three quinolizidine alkaloids. It was suggested that the N-1 nitrogen atom plays an important role in the expression of AF activity in matrine, because oxidation of the N-1 nitrogen atom (oxymatrine) resulted in total loss of the inhibitory effect on larval settlement. In addition, comparison of the structure and potency of oxysophocarpine with those of

oxymatrine revealed that the introduction of an unsaturated C-13—C-14 bond (a double bond) had no significant influence on the AF activity. On the other hand, 2′-methoxykurarinone showed much stronger inhibitory activity against barnacle settlement in comparison with matrine, oxymatrine and oxysophocarpine, indicating again the effectiveness of the approach used here in directly isolating highly AF active compounds.

In conclusion, the results confirmed that herbal plants are a valuable source of natural AF compounds, which may have the potential for the development of environmental-friendly AF technology. Furthermore, two natural products from the herb *S. flavescens*, 2′-methoxykurarinone and matrine were proven to be highly active in inhibiting barnacle settlement in a non-toxic way and could be considered as attractive candidates for incorporation into marine coatings. This study tested the AF activities of the substances against one fouling species only. Because fouling communities are comprised of a large diversity of organisms from different phyla, potentially with diverse settlement strategies, a compound able to interfere with the settlement mechanisms of barnacles may not be as effective against the adhesion of other marine fouling organisms such as bacteria, algae and mussels. Therefore, from the perspective of application in novel AF coatings, it will be of interest to determine whether these natural products have broad-spectrum efficacy against fouling species and elucidate possible AF activity in nature.

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