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Effective natural antifouling compounds from the plant *Nerium oleander* and testing

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ABSTRACT

One major challenge to commercialization of natural antifoulants is to find the effective antifouling substances from natural flora and fauna with sufficient amount available. In this study, four cardenolides, odoroside A, digitoxigenin, oleandrin and odoroside H, were isolated from a widely distributed plant *Nerium oleander* L. These four compounds and their eight analogues were then evaluated for antifouling activity against the barnacle *Balanus albicostatus* cyprids. All of the tested compounds showed a strong inhibitory activity against barnacle settlement, with EC_{50} values ranging from 0.58 to 230.67 ng ml^{-1}. Additionally, evaluation of their lethality against a non-target organism *Artemia salina* L., revealed LC_{50} values of 17.23 to above 100 μg ml^{-1}, indicating moderate to low toxicity towards *A. salina*. Furthermore, investigation of the field antifouling performance of three *N. oleander* extracts containing cardenolides by incorporation into coatings revealed significant antifouling efficiency in marine water for 30 days. These findings indicate the commercial potential for these natural antifouling products from *N. oleander* as natural antifoulants.

1. Introduction

Marine biofouling poses serious global economic problems (e.g., reduced ship speed and increased fuel consumption) and environmental risks (e.g., increased emission of greenhouse gases and dissemination of invasive foreign species) (Fletcher, 1988; Schultz, 2007; Hellio, 2010; Poloczanska and Butler, 2010; Maréchal and Hellio, 2009; Chan et al., 2014). Antifouling biocides such as organotin, copper oxide and some herbicides have been widely applied to control marine biofouling. However, there are currently bans and regulations on the use of these antifoulants due to their negative environmental impacts (Burgess et al., 2003; Bellas, 2006; Callow and Willingham, 1996; Thomas and Brooks, 2010). Hence, there is an urgent need for environmentally friendly antifouling agents. As a promising source of such alternatives, natural antifouling products have received a lot of attention.

Studies of natural antifouling products have focused on isolating antifouling active secondary metabolites from marine organisms, including marine bacteria, fungi, algae, sponges, corals, bryozoans and ascidians (Clare, 1996; Omae, 2003; Qian et al., 2009; Chen et al., 2013; Almeida and Vasconcelos, 2015). Many marine natural products with antifouling activity have been found and identified as terpenoids, steroids, saponins, alkaloids, fatty acids, amino acids, polyketides and polyphenolics (Fusetani, 2004, 2011; Omae, 2006; Qian et al., 2015). However, these are usually difficult to produce on a large-scale for commercial because they are generally not available in sufficient quantities from marine organisms and difficult to chemically synthesize at a low cost (Raveendran and Mol, 2009; Feng et al., 2009a; Peérez et al., 2014b; Qian et al., 2015). When compared with marine organisms, many terrestrial plants can be easily harvested on a commercial scale because of their wide distribution and/or mass cultivation (Feng et al., 2009b; Pérez et al., 2014a). Moreover, a few studies have confirmed the antifouling activity of extracts and compounds from terrestrial plants. *Trans*-6-, 8- and 10-shogaols, isolated from the roots of the ginger *Zingiber officinale* Roscoe, inhibited attachment of the blue mussel *Mytilus edulis galloprovincialis*, while *trans*-8-shogaol showed antifouling activity in the field (Etoh et al., 2002). Bioassay-guided isolation of acetone extract from the stem of the betel *Piper betle* led to the discovery of four piperamides with antifouling activity, and one of their synthesized analogues, 1-[1-oxo-7-(3′,4′-methylenedioxyphenyl)-6E-heptenyl]- piperidine, exhibited inhibitory activity against barnacle

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settlement (Huang et al., 2014). Incorporation of secochiliolide acid isolated from the ethanol extract of the Patagonian shrub, *Nardophyllum bryoides*, into paint inhibited settlement of macrofouling organisms for over 45 days in the sea (Pérez et al., 2014b). Taken together, these findings indicate that terrestrial plants are a valuable source of natural antifouling products.

In the reviews conducted by Rittschof (2000) and Omae (2003), the compound bufalin, isolated from the toad skin, was suggested as the most potent natural product antifoulant. In the original report by Gerhart et al. (1993), three bufadienolides (bufalin, cinobufagin, cinobufotalin) and a cardenolide (digoxin) were found to be highly antifouling active against settlement of the barnacle *Balanus amphitrite*. However, after Gerhart et al. (1993), Rittschof (2000) and Omae (2003), no studies have investigated application of these compounds in antifouling paints or antifouling activity of other analogues. One important obstacle to their application is probably their production on a large scale. Cardenolides and bufadienolides are actually well known for their cardiac activity. The backbone structure of cardenolides has a steroid nucleus and a five-membered lactone ring at C-17 (together referred to as aglycone or genin). The sugar moiety is usually attached to the C-3-OH group. Bufadienolides are different from cardenolides in structure only in that bufadienolides are characterized by a six-membered lactone ring at C-17 (Agrawal et al., 2012). Cardenolides and bufadienolides have been found in many plants, especially in Apocynaceae, including *Nerium*, *Asclepias*, and *Digitalis* (Isman, 1977; Siddiqui et al., 1997; Afolabi et al., 2011), as well as in some animals such as toads, insects and snakes (Oycke et al., 1987; Krenn and Kopp, 1998).

Nerium oleander L. (Syn, *N. indicum* Mill; *N. odorum* Soland) (Apocynaceae) is an evergreen shrub (or small tree) distributed in many subtropical and tropical areas of the world. In China, it is widely used as an ornamental plant and a medicinal plant. In the present study, we isolated four cardenolides from this plant and evaluated their antifouling activity against settlement of *B. albicostatus* cyprids. Additionally, eight analogues were also tested against *B. albicostatus* to obtain information regarding structure-activity relationships. All the compounds were evaluated for toxicity toward a non-target organism, *Artemia salina*. Furthermore, field tests of the *N. oleander* extracts containing cardenolides were conducted to confirm their antifouling potency in the marine environment.

2. Materials and methods

2.1. Extraction, isolation and identification of active compounds from N. oleander

2.1.1. Extraction and isolation

The stems of *N. oleander* were collected from Dadeng Island, Fujian Province, China in July 2013. The species was identified by Professor Z.J. Li, School of Life Sciences, Xiamen University. The *N. oleander* stems (7.25 kg) were air-dried, powdered mechanically and extracted with methanol three times. The combined extracts (258 g) were then suspended in 90% methanol (CH_3OH, 1 l) and further extracted with petroleum ether (1 l). The remaining solution was evaporated under reduced pressure and resuspended in distilled water (1 l), after which it was successively partitioned with equal volumes of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and n-butanol. The resultant five fractions, including the residual aqueous fraction, were dried under reduced pressure and then tested for antifouling activity. The active CH_2Cl_2 fraction (44.85 g) was separated on a silica gel column eluted with petroleum ether-EtOAc (5:1 then 4:1 then 3:1) to produce 11 fractions (F1 − F11). The active fractions F7 and F9 were further subjected to column chromatography. F7 (1.73 g) was isolated by silica gel column chromatography and elution with $CHCl_3$-CH_3OH (60:1 followed by 40:1, 20:1 and then 10:1) to give eight sub-fractions (F7.1 − F7.8). F7.4 (112.5 mg) was subjected to a Sephadex LH-20 column and eluted with methanol to give two fractions (F7.4.1 − F7.4.2). The fraction F7.4.1 (60.0 mg) was purified by high performance liquid chromatography (HPLC) using a C18 column (5 μm, 10 × 250 mm, ThermoScientific, USA), then eluted by a gradient of CH_3OH-H_2O (3:7–8:2) at a flow rate of 2.0 ml min^{-1} to yield compound **1** (30.0 mg, white powder), and the same method was used to purify F7.4.2 (24.3 mg) to yield compound **2** (5.8 mg, white powder). Another subfraction, F7.5 (1032.0 mg), was subjected to chromatography on a silica gel column and eluted with petroleum ether-EtOAc (4:7 followed by 4:9, 1:3 and then 1:5) before being further purified by the same HPLC C18 column as mentioned above and gradient elution with CH_3OH-H_2O (4:6–8:2) to yield compound **3** (7.4 mg, white powder). Fraction F9 (230 mg) was subjected to column chromatography using silica gel eluted with $CHCl_3$-CH_3OH (50:1 followed by 20:1, 5:1 and then 1:1) before being further purified on a silica gel column eluted with n-hexane-acetone (2:3 followed by 1:2, 1:5 and then 1:9) to obtain compound **4** (13.8 mg, white powder).

2.1.2. Identification of compounds

The structures of the compounds were identified based on NMR and MS spectral data. The NMR spectra were obtained in deuterochloroform ($CDCl_3$) or deuteromethanol (CD_3OD) on a Bruker Avance II 400 instrument (^{1}H-NMR, 400 MHz; ^{13}C-NMR, 100 MHz) with tetramethylsilane (TMS) as the internal standard. The MS data were measured in positive ion mode on a Bruker ESI-Q-TOF mass spectrometer.

2.2. Bioassay for antifouling activity against the barnacle Balanus albicostatus

B. albicostatus adults were collected from the intertidal zone in Xiamen, Fujian Province, China. Upon immersion in seawater, the adults released the naupliar larvae, which were cultured to cyprids as previously described by Feng et al. (2009a). The assay against settlement of barnacle cyprids was conducted as described by Hellio et al. (2005) and Kitano et al. (2004). The compounds isolated from *N. oleander* were dissolved in methanol and applied to glass Petri dishes (6 cm diameter). After complete evaporation of the solution, 10 ml of filtered (0.22 μm) seawater (FSW) and 30 cyprids were added to each dish. There were three replicates for each treatment and the FSW control. The Petri dishes were incubated in the dark at 25 °C for 72 h, after which the number of cyprids that had settled, died or were still swimming was counted under a stereomicroscope.

2.3. Bioassay for toxicity against the brine shrimp Artemia salina

The bioassay for toxicity against *A. salina* was conducted using the Artoxkit M procedure (Artoxkit, 1990), with slight modification. *Artemia* cysts were purchased from Wudi Aijia Pet Aquarium Co., Ltd. (Binzhou, China) and incubated in FSW with continuous illumination (3000–4000 lux) and aeration at 25 °C. After 24 h, the hatched larvae were transferred to fresh FSW and incubated for another 24 h. Next, *Artemia* nauplii of instar stages II–III were collected for use in the bioassay. The bioassay was conducted using 24-well plates. The compounds were dissolved in dimethyl sulfoxide (DMSO). A volume of 10 μl of each compound solution, 1.99 ml FSW and 10–20 nauplii were added into each well of a 24-well plate. Wells containing 0.5% DMSO in FSW (*v/v*) served as controls. There were six replicates for each treatment and the solvent control. After 24 h of incubation in darkness at 25 °C, the dead nauplii in each well were counted.

2.4. Evaluation of antifouling activity and toxicity of eight analogues

In this study, the antifouling activity and toxicity of eight analogues of the compounds we isolated from *N. oleander* (four cardenolide compounds) were tested. Seven bufadienolides (cinobufagin, resibufogenin, gamabufotalin, arenobufagin, telocinobufagin, bufotalin

Fig. 1. Chemical structures of four compounds isolated from *Nerium oleander*.

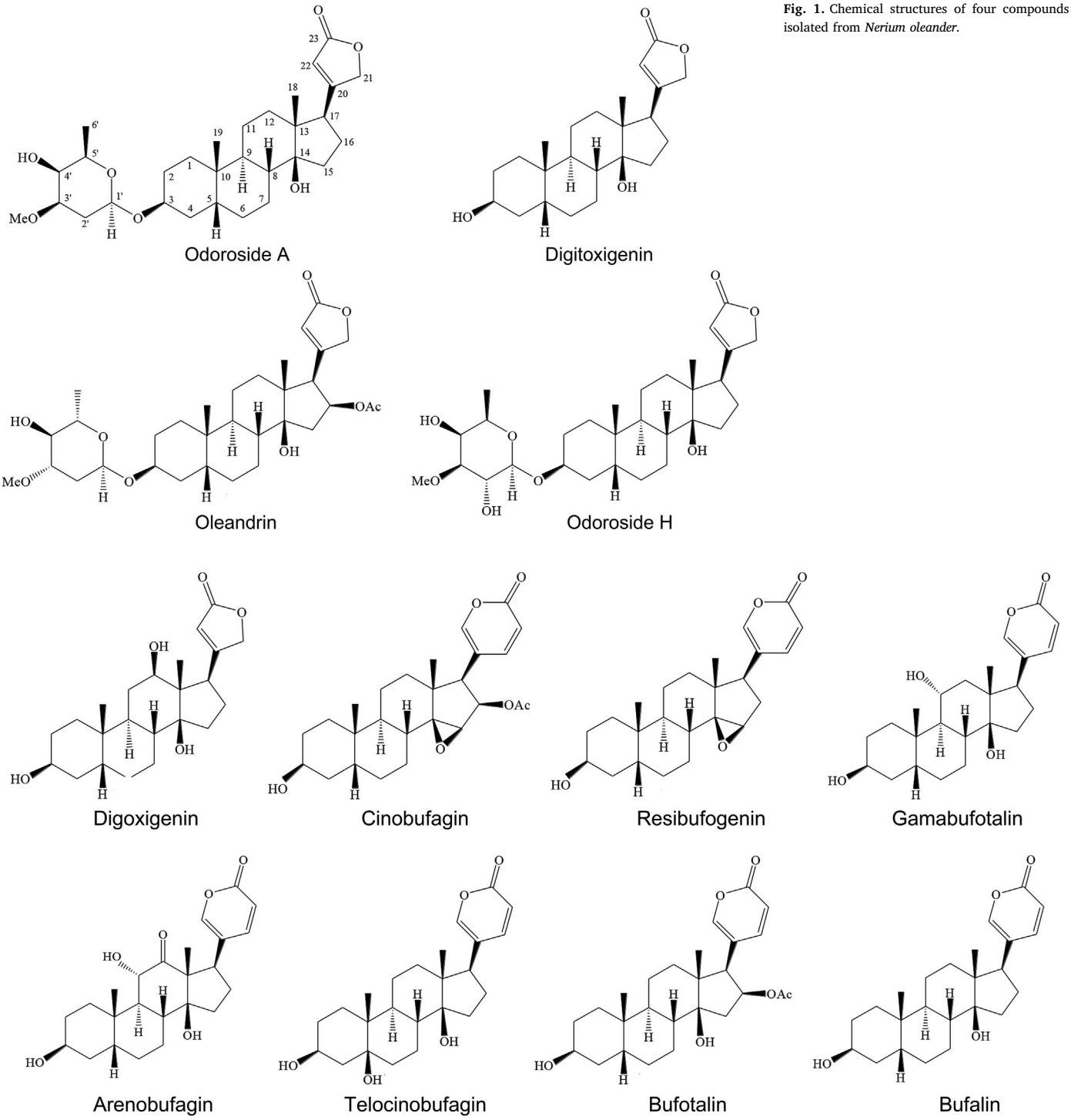

Fig. 2. Chemical structures of eight analogues of the compounds isolated from *Nerium oleander* in this study.

and bufalin) with purity > 95% were provided by Dr. Qiu (School of Pharmaceutical Sciences, Xiamen University, China), whose research group isolated these compounds from the skin of *Bufo bufo gargarizans* (Zhao et al., 2011; Qiu et al., 2014). Digoxigenin with purity > 95% was purchased from Sigma-Aldrich. The chemical structures of these eight compounds are shown in Fig. 2. Compounds were tested against *B. albicostatus* for antifouling activity and against *A. salina* for toxicity as described above.

2.5. Field tests

2.5.1. Preparation of N. oleander extracts

Because extracts usually cost much less than preparations of pure compounds and are therefore more acceptable for commercial use, we conducted a field investigation of the antifouling potency of *N. oleander* extracts containing cardenolides. Three extracts from *N. oleander*, EI, EII and EIII, were tested. Extract EI was the methanol extract from the stems of *N. oleander* and EII was the CH_2Cl_2-soluble fraction from EI. Extracts EI and EII were prepared as described in the Extraction and isolation section, while extract EIII was prepared as described in Wang

Table 1
Antifouling activity of the compounds isolated from *Nerium oleander* and their eight analogues against the barnacle *Balanus albicostatus*.

Compound	EC_{50} (ng ml^{-1})	LC_{50} (ng ml^{-1})	LC_{50}/EC_{50}
Odoroside A	4.60	4.70	1.02
Digitoxigenin	32.09	64.93	2.02
Oleandrin	15.15	16.32	1.08
Odoroside H	23.88	36.03	1.51
Digoxigenin	230.67	568.07	2.46
Cinobufagin	0.58	1.19	2.05
Resibufogenin	69.80	168.99	2.42
Gamabufotalin	3.02	7.45	2.47
Arenbufagin	2.24	3.16	1.41
Telocinobufagin	2.02	2.24	1.11
Bufotalin	1.21	1.97	1.63
Bufalin	0.69	1.53	2.22

Table 2
Toxicity of the compounds isolated from *Nerium oleander* and their eight analogues against the brine shrimp *Artemia salina*.

Compound	LC_{50} (μg ml^{-1})
Odoroside A	17.23
Digitoxigenin	> 100
Oleandrin	28.07
Odoroside H	57.07
Digoxigenin	> 100
Cinobufagin	> 100
Resibufogenin	37.90
Gamabufotalin	70.99
Arenobufagin	39.50
Telocinobufagin	> 100
Bufotalin	> 100
Bufalin	> 100

et al. (2006) whose aim was to obtain extract rich in cardenolides from *N. indicum* (syn. *N. oleander*), with slight modification. Briefly, for preparation of EIII, the leaves and stems of *N. oleander* were crushed mechanically and extracted exhaustively with 80% aqueous alcohol three times at room temperature (25 °C). The combined extracts were then concentrated under reduced pressure and partitioned between diethyl ether and H_2O. The aqueous layer was subsequently added into a saturated solution of lead subacetate, intensively mixed and then filtered to remove deposits. To remove the excess lead, H_2S was pumped into the remaining solution, which was then mixed and filtered to remove deposits. The solution was subsequently evaporated under reduced pressure to give EIII. The presence of cardenolides in extracts EI, EII and EIII was confirmed by HPLC.

2.5.2. Paint preparation

In the test with the extracts EI and EII, paints were prepared by mixing the ingredients using a high-speed disperser. The composition of ingredients in the matrix paint, expressed as weight percentage, was as follows: 14.8% colophony, 65.8% polyacrylic resin, 7.7% Fe_2O_3, 1.4% bentonite and 10.3% mixed solvent (coal-tar: n-butanol: dimethyllbenzene = 14:3:13). This mixture was dispersed for 2 h, after which extract EI or EII was added into the mixture at 10% (*w/w*), then dispersed for another 10 h to obtain the paint. The control paint was made of the matrix paint without antifouling substances and obtained after 12 h of dispersion of the mixed ingredients of the matrix paint. In the test of extract EIII, the paints were prepared as described above, except that extract EIII was added into the matrix paint at 20% (*w/w*) as treatment paint. The control paint consisted of matrix paint without antifouling substances.

2.5.3. Submerged assays

In the assay with the extracts EI and EII, the prepared paints were brushed onto sandblasted epoxy panels, with the painted area of 20 × 7 cm for each paint. Three layers of paint were then applied and allowed to dry for 24 h between each application, resulting in a final dry film thickness of ca 150 μm. Panels coated with the matrix paint alone were used as controls. The panels were hung on a floating raft at a depth of 1 m in Xiamen Bay, China (24°33′ N, 118°10′ E) on August 25, 2014 and submerged for one month. At the end of the test period, barnacles were the only macrofoulers observed on the panels; therefore, the number of barnacles on each panel was counted and their density was calculated. In the assay of extract EIII, the paints were applied onto the sandblasted epoxy panels using the method as described above. Panels coated with matrix paint alone were used as the control. The panels were then immersed at a depth of 1 m at a fish farm in Lingshui Bay, China (18°25′ N, 109°59′ E) on January 30, 2016 and submerged for one month. At the end of the experimental period, tubeworms were the only macrofouling organisms on the panels. Because the tubeworms were too crowded to count the individuals, the panels were photographed and the percentage of the surface colonized by the fouling organisms on each panel was calculated using Adobe Photoshop 7.0. Both assays were performed in six replicates.

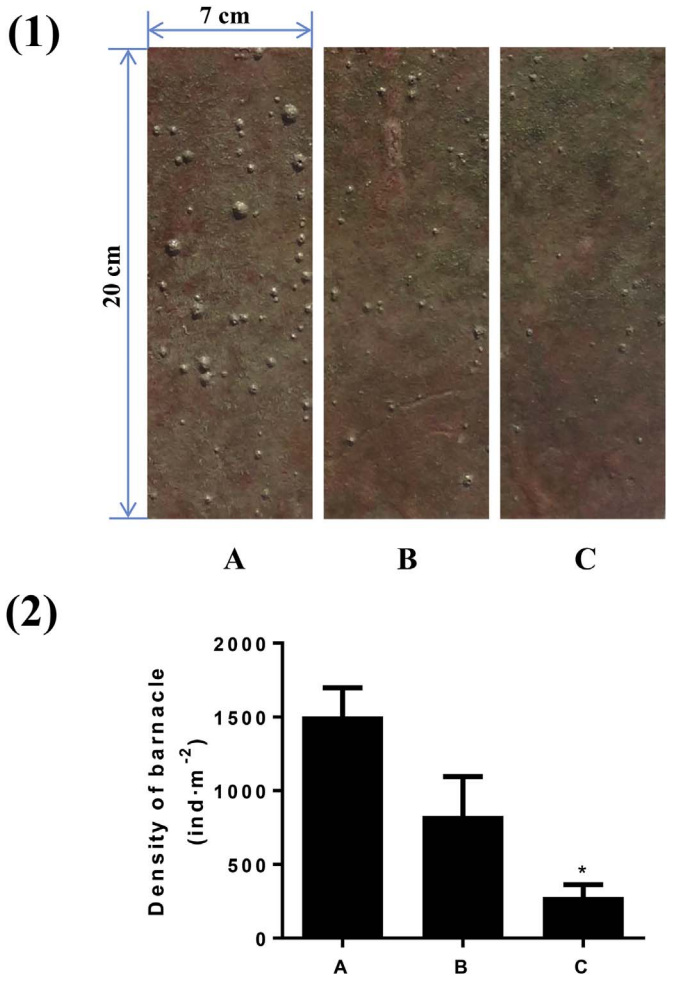

Fig. 3. Field tests of extracts EI and EII. (1) Test panels after submersion in seawater for one month in Xiamen Bay. The spots on the panels represent fouling by barnacles. (2) Density of barnacles on the test panels. Data shown are the means of six replicates + standard error. *Asterisks* above the bars indicate significant differences from the control ($P < 0.05$). A: coated with matrix paint only (control); B: coated with paint containing extract EI; C: coated with paint containing extract EII. Preparation of extracts EI and EII was conducted as described in the Materials and Methods section.

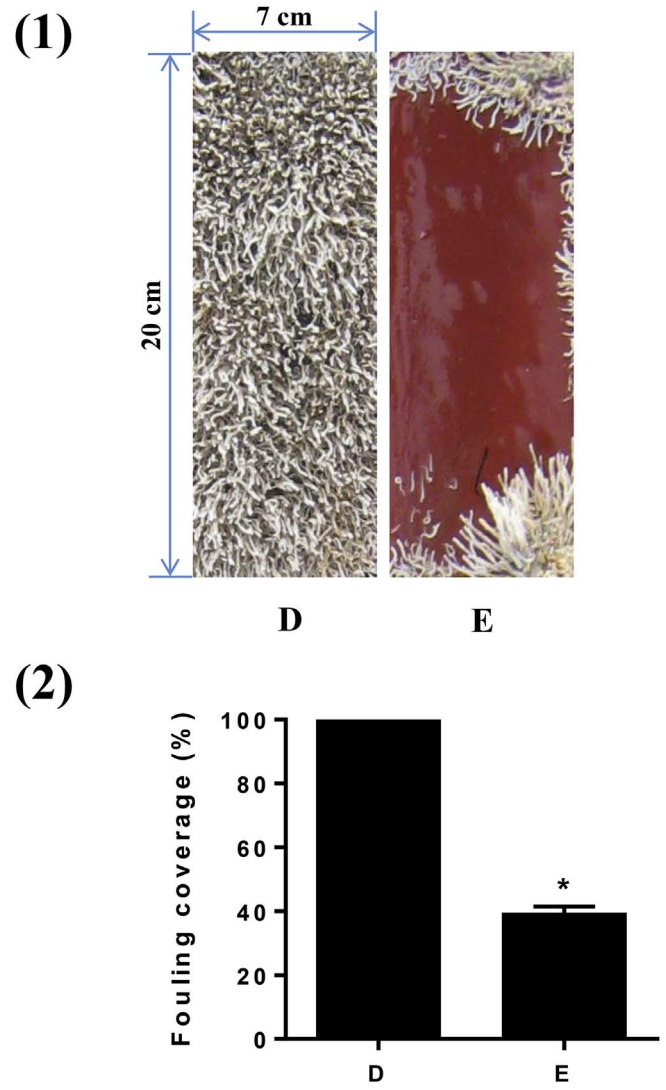

Fig. 4. Field tests of extract EIII. (1) Test panels after submersion in seawater for one month in Lingshui Bay. (2) Fouling coverage on the test panels. Data shown are the means of six replicates + standard error. *Asterisks* above the bars indicate significant differences from the control ($P < 0.05$). D: coated with matrix paint only (control); E: coated with paint containing extract EIII. The preparation of extract EIII was conducted as described in the Materials and Methods section.

2.6. Statistical analysis

In the bioassays, antifouling activity, expressed as the EC_{50} value (the concentration that reduced barnacle larval settlement by 50% relative to the control), and the toxicity, expressed as the LC_{50} value (the concentration that resulted in 50% mortality of *B. albicostatus* cyprids or *A. salina* nauplii), were estimated using the Spearman-Karber method (Hamilton et al., 1977, 1978; Reichelt-Brushett and Michalek-Wagner, 2005). For the field tests, differences in the barnacle density or fouling coverage between treatments and the controls were analyzed by Student's *t*-test. The significance level was defined as $P < 0.05$.

3. Results

3.1. Isolation and identification of active compounds from N. oleander

Four compounds were isolated from the CH_2Cl_2 fraction of the methanol extract of *N. oleander* stems in the present study. The ^{1}H-NMR, ^{13}C-NMR and ESI-MS data of the four compounds are shown in the supplementary file. Based on their spectral data and comparison with those in the literature (Abe and Yamauchi, 1978; Abe et al., 1996; Cruz et al., 1977; Cabrera et al., 1993), the compounds were identified as odoroside A, digitoxigenin, oleandrin and odoroside H, which are all cardenolides. Their chemical structures are shown in Fig. 1.

3.2. Antifouling activity of compounds from N. oleander and their analogues

As shown in Table 1, the four cardenolides isolated from *N. oleander* and their eight analogues were antifouling active against settlement of the barnacle *B. albicostatus*, with EC_{50} values all below 250 ng ml^{-1}. The most pronounced antifouling activity against *B. albicostatus* was found in cinobufagin, which had an EC_{50} of 0.58 ng ml^{-1}. It should be noted that the EC_{50} of bufalin (0.69 ng ml^{-1}) is only slightly lower than that of cinobufagin. Among the twelve compounds tested, digoxigenin exhibited the lowest antifouling activity, with an EC_{50} of 230.67 ng ml^{-1}. The tested compounds also showed various toxicity to *B. albicostatus* cyprids, with the most toxic compound being cinobufagin (LC_{50} 1.19 ng ml^{-1}) and the least toxic being digoxigenin (LC_{50} 568.07 ng ml^{-1}). The LC_{50}/EC_{50} ratios of these twelve compounds were low and close to each other (around 1 or 2, Table 1).

3.3. Toxicity of compounds from N. oleander and their analogues against A. salina

As shown in Table 2, among the twelve compounds tested, six (digitoxigenin, digoxigenin, cinobufagin, telocinobufagin, bufotalin and bufalin) had an LC_{50} above 100 μg ml^{-1} against *A. salina*, indicating their toxicity to this non-target organism is very low. The other six compounds (odoroside A, oleandrin, odoroside H, resibufogenin, gamabufotalin and arenobufagin) exhibited moderate toxicity to *A. salina*, with LC_{50} values ranging from 17.23 to 70.99 μg ml^{-1}.

3.4. Field tests of N. oleander extracts

Here, three extracts containing cardenolides from *N. oleander* were subjected to field tests. As shown in Fig. 3, barnacles appeared on test panels after submersion for one month in Xiamen Bay. A lower barnacle density was observed on panels coated with extract EI than the control panels, but this difference was not statistically significant ($P = 0.072$). In the case of panels coated with the EII extract, the mean barnacle density (275.22 ± 86.74 ind m^{-2}) was significantly lower than that of control panels (1499.08 ± 198.89 ind m^{-2}, $P < 0.05$), indicating the antifouling potency of extract EII in the field. As shown in Fig. 4, tubeworms covered the entire surface of the control panels after immersion for one month in Lingshui Bay. In comparison, significantly lower fouling coverage ($P < 0.05$) was observed on panels coated with extract EIII, suggesting the effective antifouling performance of this extract in the marine environment.

4. Discussion

Extracts and secondary metabolites from *N. oleander* have been found to possess various insecticidal, antimicrobial, cardiotonic, anticancer and antiplatelet aggregation bioactivities (Elshazly et al., 2000; El Sawi et al., 2010; Adome et al., 2003; Rashan et al., 2011; Begum et al., 1996; Chen et al., 2013; Chan et al., 2014). It was previously reported that the crude extracts of two Apocynaceae plants, *N. indicum* and *Thevetia peruviana*, were significantly toxic to *B. albicostatus* nauplii (Lin and Lu, 2008). This study identified four antifouling active cardenolides (odoroside A, digitoxigenin, oleandrin and odoroside H) from *N. oleander*. Cardenolides and bufadienolides, which are similar in chemical structure, have been used in clinical practice for the treatment of heart failure and atrial arrhythmia (Prassas and Diamandis, 2008), and their antibacterial, anticancer and anti-inflammatory activities have also been reported (Huq et al., 1999; Menger et al., 2012; Lee et al.,

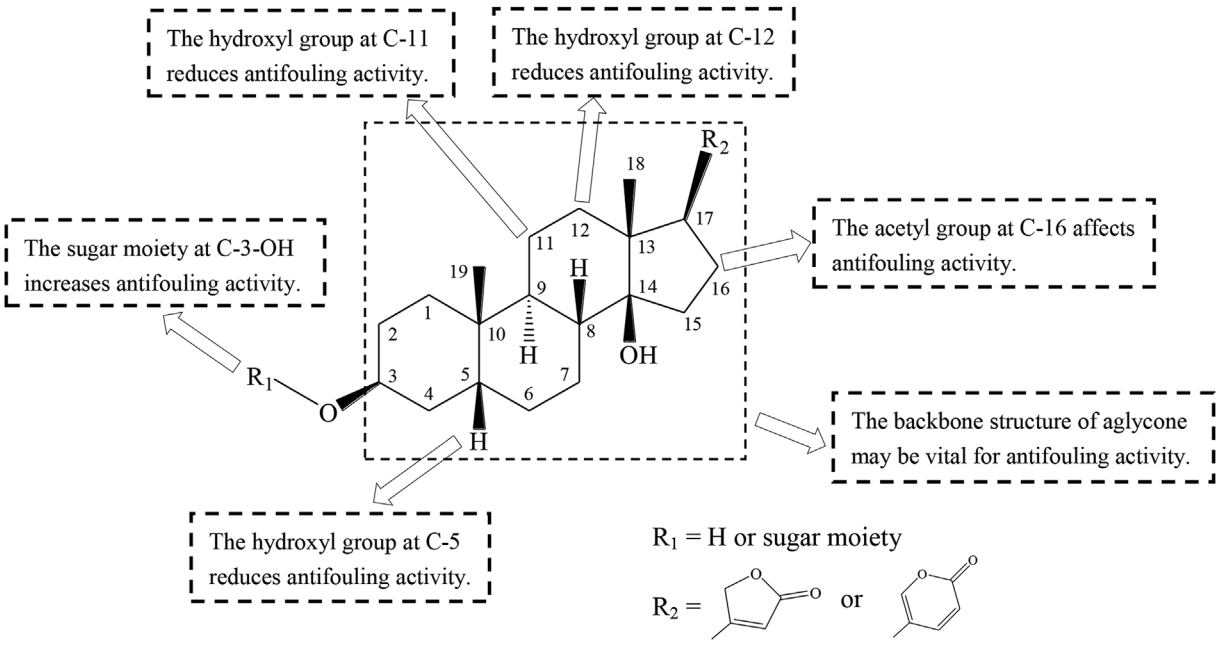

Fig. 5. The structure-antifouling activity relationships of the steroid compounds tested here.

2013; Zhao et al., 2007). Furthermore, they were found to be toxic to insects and snails (Scudder and Duffey, 1972; Dobler et al., 2011; Hussein et al., 1994; Al-Sarar et al., 2012). In the present study, five cardenolides and seven bufadienolides were shown to have high antifouling activity against the barnacle *B. albicostatus*, with EC_{50} values all much lower than 25 μg ml^{-1}, which is the standard requirement established by the US Navy program as a potency criterion for natural antifoulants (Rittschof, 2001). The LC_{50}/EC_{50} ratio, also known as the therapeutic ratio, provides insight into the mechanism of action of compounds (Rittschof et al., 1992). For a compound, a high LC_{50}/EC_{50} ratio indicates that it inhibits settlement in a non-toxic way, while a low LC_{50}/EC_{50} ratio indicates the inhibition of settlement occurs through a toxic pathway (Rittschof et al., 1992; Lau and Qian, 1997). The LC_{50}/EC_{50} ratios of the compounds tested here were only around 1 or 2, suggesting that they inhibited barnacle settlement via a toxic mechanism.

The cardiac activity of cardenolides and bufadienolides has been attributed to their ability to inhibit the animal enzyme Na^+/K^+-ATPase (Steyn and Van Heerden, 1998; Melero et al., 2000). Furthermore, cardenolides in Apocynaceae plants act as poisons and emetics against herbivorous insects, playing an important role in chemical defense of the plants (Scudder and Duffey, 1972; Agrawal et al., 2012). The mode of action for cardenolides on insects was discussed in Agrawal et al. (2012) and it also involved the inhibitory effect on Na^+/K^+-ATPase. Since barnacles and insects both belong to the phylum Arthropoda, knowledge of their mechanism of action on insects may provide some insights for understanding the mechanism of antifouling activity of these tested compounds against barnacles.

Based on the similar chemical structures and different antifouling activities of the compounds tested here, some information about the structure activity relationship (SAR) could be obtained (Fig. 5). All of the tested compounds exhibited high activity against barnacles, indicating that the backbone structure of aglycone may be vital for antifouling activity, regardless of whether the unsaturated lactone ring at C-17 is five or six membered. However, the presence of the unsaturated lactone ring may have played a role in antifouling activity of these compounds. The unsaturated lactone has been suggested as an important feature of a few antifouling compounds (Clare et al., 1999; Xu et al., 2010), such as halogenated furanones isolated from the red alga *Delisea pulchra* (De Nys et al., 1995), and alkylating butenolides isolated from a marine *Streptomyces* strain (Xu et al., 2010). In addition, based on comparison of the EC_{50} values of digitoxigenin and odoroside A and considering that the only difference between their structures is the lack of a sugar moiety in digitoxigenin, it was suggested that the presence of a sugar moiety attached to the C-3-OH group increased the antifouling activity. This was supported by similar analysis of the structure–activity relationship of digitoxigenin and odoroside H. For digitoxigenin and digoxigenin, the only structural difference between these two compounds is the presence of the hydroxyl group at C-12 in digoxigenin, but the EC_{50} of digoxigenin is much higher than that of digitoxigenin, indicating that the hydroxyl group at C-12 reduces antifouling activity. A similar case was also found in comparison of gamabufotalin and bufalin. The only structural difference between them is the presence of the hydroxyl group at C-11 in gamabufotalin. As revealed by the higher EC_{50} of gamabufotalin than that of bufalin, the presence of the hydroxyl group at C-11 in gamabufotalin reduces its antifouling activity. Similar analysis of the structure–activity relationship of telocinobufagin and bufalin also indicated that the presence of the hydroxyl group at C-5 in telocinobufagin reduces antifouling activity. The results of the above analysis suggest that the presence of a hydroxyl group at some positions of the steroid backbone has a reducing effect on antifouling activity. Furthermore, cinobufagin and resibufogenin have a very similar structure, with an acetyl group present at C-16 in cinobufagin, but absent from resibufogenin. Here cinobufagin exhibited much higher antifouling activity than resibufogenin. However, the same structural difference was found between bufotalin and bufalin (i.e., the presence of the acetyl group at C-16 in bufotalin but the absence of this group in bufalin), and bufotalin showed lower antifouling activity than bufalin. This inconsistency suggested that the acetyl group at C-16 may enhance or reduce antifouling activity, and the effect of this group on antifouling activity may depend on other substituent group(s) of the compounds as there is an epoxy group at C-14 and C-15 in cinobufagin and resibufogenin, but a hydroxyl group at C-14 in bufotalin and bufalin. Moreover, the structures of gamabufotalin and arenobufagin differ in the presence of the carbonyl group at C-12 in arenobufagin, and there is only slight difference in EC_{50} values of these two compounds, indicating that the carbonyl group at C-12 does not influence antifouling activity strongly.

Although the compounds investigated here all exhibited low LC_{50} values against barnacle cyprids and low LC_{50}/EC_{50} ratios, indicating

high toxicity to this target organism, their toxicity toward the non-target organism *A. salina* was very low or moderate. Furthermore, the LC_{50} values of these twelve compounds against *A. salina* were all much higher than those of the three commercial antifoulants, tributyltin (already banned by IMO), Irgarol and copper (LC_{50} 4.14 × 10^{-5}, 1.62 and 0.28 μg ml^{-1} respectively, Panagoula et al., 2002, Zulkifli et al., 2014). Therefore, there is potential for them to be used as environmentally friendly antifouling agents. However, their toxicity toward other non-target marine organisms and degradation in the marine environment should be investigated in future studies prior to their widespread application.

Bufalin was remarkable for its high antifouling activity against barnacle (Rittschof, 2000; Omae, 2003). Here we found four analogues of bufalin (four cardenolides), with comparative antifouling activity, from the plant *N. oleander*. *N. oleander* is widely distributed, suitable for mass culture and could be easily collected, which is hugely advantageous for commercial application of its active substances. Furthermore, the present study confirmed that the cardenolides-containing extracts of *N. oleander* were antifouling effective in the field, indicating the potential for a low-cost method of exploiting *N. oleander* since it is much easier to obtain extracts than to prepare compounds. The test results of the three extracts also suggested that there are options for preparing effective extracts via different procedures. Given the above-mentioned advantages for commercial application of extracts from *N. oleander* in novel antifouling coatings, there is great potential for their practical application.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2017.11.022.

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