

Insecticidal Activity and Insect Repellency of Four Species of Sea Lily (Comatulida: Comatulidae) From Taiwan

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Abstract

The methanol and ethyl acetate (EA) extracts of four species of sea lily (*Himerometra magnipinna*, *Comaster multifidus*, *Comanthina* sp., and *Comatella maculata*) were evaluated for their insecticidal activity against Yellow-fever mosquito larvae (*Aedes aegypti*) and their repellency against adult Asian Tiger mosquitoes (*Aedes albopictus*). The 24-hr minimum inhibition concentration (MIC) data revealed that the extracts from *H. magnipinna* and the *C. maculata* were the most active, killing mosquito larvae at 12.5 ppm. The toxicity of the extracts from these four sea lilies in descending order was *H. magnipinna* (12.5 ppm), *C. maculata* (12.5 ppm), *C. multifidus* (100 ppm), and *Comanthina* sp. (200 ppm). Furthermore, no significant difference in toxicity was found using either EA or methanol as the extraction solvent. The MIC at 12.5 ppm is promising as an insecticide lead. The repellency study results show that EA is a better solvent for one species (*H. magnipinna*), but the methanol is a better solvent overall. The repellency of these sea lily extracts in descending order was *Comanthina* sp. MeOH (ED₅₀ at 0.32%), followed by *H. magnipinna* EA (ED₅₀ at 0.38%), *C. multifidus* MeOH (ED₅₀ at 0.57%), *C. maculata* MeOH (ED₅₀ at 0.76%), *C. multifidus* EA (ED₅₀ at 1.25%), and *H. magnipinna* MeOH (ED₅₀ at 1.67%). A compound with ED₅₀ < 0.5% is considered to be a promising repellent. Among the studied sea lilies, both *Comanthina* sp. and *H. magnipinna* have potential to be further developed as mosquito control agents due to their favorable toxicity and repellency.

Key words: *Aedes aegypti*, *Aedes albopictus*, insecticidal activity, insect repellency, sea lily, Taiwan

Natural products (NP) have been and continue to be an excellent source of inspiration for new insect control products. Research on NP as pest control agents has recently become popular, as the synthetic alternatives have drawbacks chemical resistance on target insect pests, emergence of secondary insect pests, and toxicity to human beings and the environment (Geiger 1993). The majority of natural insecticides are from terrestrial plants; for instance, pyrethrins come from the chrysanthemum plant, and azadirachtin is derived from the neem tree. No commercial marine insecticides yet exist, although some marine organisms are known for their toxicity to cell lines. For example, trabectedin extracted from sea slug, *Ecteinascidia turbinata* (Rinehart 2000), and kahalalide F from tunicate (also known as sea squirt), *Elysia rufescens* (Suárez et al. 2003) have been clinically proven to be effective against several cancers, and both have been launched as cancer fighting drugs.

Few studies have been performed on the application of marine organisms as pest control agents, even though their toxicities are well known. Several species of sea squirt have been reported as

having strong insecticidal activity (LC₅₀ ranged from 0.02 to 0.03 µg/ml) against two species of adult mosquitoes (Zeti et al. 2001) and strong repellency (ED₅₀ ranged from 0.008 to 0.0746 mg/cm²) against four species of mosquitoes (Zeti et al. 2002). A quick insecticide bioassay for NP at National Taiwan University included sea lily extract, which against expectations killed all mosquito larvae at 10 µg/ml (Chio 2007). The same sea lily sample was later reported to repel mosquitoes with an ED₅₀ value of 1.02% (Chio and Yang 2008).

Marine biodiversity is high in Taiwan. Recent reports suggest that Taiwan has ~10% of the total marine species in the world, despite having only 0.0003% of the Earth's land mass (<http://taibnet.sinica.edu.tw>). Sea lilies are common in Taiwan, but have not been widely studied in this region. The biological activities of sea lilies in particular have not been practically studied investigated at all (Chen 1986). Sea lilies or feather-stars are marine invertebrates that make up the class Crinoidea of the echinoderms (phylum Echinodermata). Most crinoids have more than five arms and have a stem used to

attach themselves to a substrate, but many become free-swimmers as adults. They live both in shallow water and in depths as great as 600 m. Comasteridae is a family of crinoids, and is distinguished from other crinoids by the position of the mouth. The mouth in comasterids is offset from the centre, while it is centrally placed in other crinoids. Comasterids prefer shallower waters than most other crinoids, and often form the dominant components of the crinoids fauna at depths of <50 m. Himerometridae is another crinoid family, and includes six genera known from the tropical Indo-west Pacific region. This study explored the toxicity and insect repellency of three species of Comasteridae and one species of Himerometridae collected in Hsiao-Lu-Chiao, an island off southwest Taiwan, and examined their potential as pest control agents.

Material and Methods

Sample Collection and Preparation

Samples of four sea lily species were collected from Hsiao-Lu-Chiao (120° 22'E and 22° 21'N) off the southwest coast of Taiwan by SCUBA divers. They were identified by morphology, size, and color, according to keys and references of Rasmussen (1978) and Chen (1986). Samples were washed with distilled water to remove sand, sediment, and other contaminants. The extraction procedure is similar to that reported by Chou et al. (2008). First, samples were frozen-dry, dehydrated, and then homogenized into fine powder. Four subsamples of 10 mg each of dry sample were mixed with 50 ml methanol (MeOH) or ethyl acetate (EA) for ~30 min at 30°C. Samples were kept in a dark place while being stirred. After mixing for 30 min, samples were filtered, and the MeOH or EA extracts were collected. Approximately 200 ml of extracts was obtained. These extracts were then lyophilized, pulverized, labeled, and stored in a -20°C freezer until assay.

Insect Preparation

A laboratory colony of the Yellow-fever mosquito (*Aedes aegypti bora*) and the Asian tiger mosquito (*Aedes albopictus*) was used in this study. Both colonies had been maintained in the Department of Entomology, National Taiwan University (NTU), Taiwan, for over 10 yr using methods described by Gerber et al. (1994). For the toxicity study, a paper tower with the Yellow-fever mosquito eggs was placed in a small container with distilled water and trace amounts of yeast. Larvae hatched from the eggs within an hour at room temperature. The newly hatched larvae were fed with ground-up dog food, and developed to third instar larvae in ~4 d. The third instar larvae of this Yellow-fever mosquito were then used for the toxicity assay. Asian tiger mosquito adults were used for the mosquito repellency assay. They were fed with sugar water and blood meals daily. However, adult mosquitoes fasted for 48 hr before the repellency study.

Animal Preparation

Living mice (random stock mouse, ICR strain) were used to provide blood meals for both species of mosquitoes. Mice were purchased from the Laboratory Animal Center (LAC) of NTU, Taiwan. The animal care at his LAC was carried out in strict accordance with international standards. The LAC received a full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in June 2007 (www.nlac.org.tw/english/).

Toxicity assays

The toxicity study used Yellow-fever mosquito larvae. This species is commonly used by the World Health Organization for insecticide evaluation (WHO 1981). Sea lily samples were weighed out and mixed with appropriate amounts of methanol resulting in a 1% stock solution. These 1% solutions were then serially diluted (1:1) with methanol to 12 concentrations ranging from 10,000 to 4.9 ppm. The toxicity assays were performed in a 96-well microtitre plate with third instar larva of *A. aegypti* according to the procedure described by Chio (2007). Exactly, 100 µl of sea lily sample was transferred to a 96-well plate and allowed to evaporate to complete dryness by a heat block. Samples were reconstituted with 100 µl of 0.01% Keflin solution followed by another 100 µl of water containing several third instar mosquito larvae. Methanol and 0.5% permethrin were adopted as negative and positive controls, respectively. Larvae mobility and mortality 24-hr post-treatment were recorded with “+” for all kill, “±” for partial kill, and “-” for no kill. Four replicates were adopted for each treatment group. Any samples that completely killed the mosquitoes at 156 ppm or higher concentrations were retested at lower concentrations. Another serial dilution (1:1) starting at 200 ppm was used for the retesting. Methanol and 0.5% permethrin were again adopted as negative and positive controls for the retesting. The minimum concentration that provided the complete control of the mosquito larvae was defined as the minimum inhibition concentration (MIC). The MIC method has been demonstrated to match well with that of LC₅₀ by comparison side-by-side with the LC₅₀ values of five common mosquito larvicides (Chio 2007). This study adopts the MIC method instead of the LC₅₀ method, because MIC is simpler than LC₅₀, and can handle higher sample volumes.

Mosquito Repellent Assay

Adult Asian tiger mosquitoes were adopted in the mosquito repellency test. The tiger mosquito is one of the most common mosquito species adopted in chemical repellency studies (Novak and Lampman 2001). The principle of this assay is based on the behavior of female mosquitoes that require blood meal before laying eggs. The test procedure was similar to that described by Chio and Yang (2008). A fiber glass window screen with mesh (2.5 mm by 2.5 mm) was adopted to build a blood-meal tube. Two identical pieces of this window screen were cut (at 5 cm by 12 cm) each. One screen was covered with utility tape, and another screen was applied with 0.5 ml of tested solution. A commercial mosquito repellent named OFF (15% DEET, *N,N*-diethyl-*m*-toluamide, manufactured by S.C. Johnson Company, Racine, WI, USA) was used as positive control in this study. This repellent was sprayed onto the test area until run off. Methanol applied the same way was adopted as negative control. After the treated screen was air-dried for 4 hr, the treated screen and the tape-cover screen were clamped together in three sides to make a “tube”. A mouse that was first wrapped with a thin cheese cloth was then loaded into the “tube”. The tube was closed with another clamp to form a blood-meal tube. This blood-meal tube containing the live mice was placed in a mosquito rearing container with ~300 mosquitoes (aged 5–14 d) which had been starved for 2 d. Numbers of mosquitoes that landed on the screen were recorded at 1 min after the blood-meal tube was placed in the mosquito rearing container. Four replicates were used in this study. The percent repellency was determined by the formula described by Weaving and Sylvester (1967).

$$R = (1 - T/C) \times 100,$$

where *R* is percentage repellency, *T* is number of mosquitoes land on treated screens, and *C* is number of mosquitoes land on methanol control screen.

Table 1. MICs (PPM) of sea lily against *A. aegypti* larvae

Samples	PPM											
	200.00	100.00	50.00	25.00	12.50	6.25	3.12	1.56	0.78	0.39	0.20	0.10
<i>H. magnipinna</i> , EA	+	+	+	+	+	(±)	-	-	-	-	-	-
<i>C. multifidus</i> , EA	+	+	(±)	-	-	-	-	-	-	-	-	-
<i>H. magnipinna</i> , MeOH	+	+	+	+	(±)	-	-	-	-	-	-	-
<i>C. multifidus</i> , MeOH	+	+	-	-	-	-	-	-	-	-	-	-
<i>Comanthina</i> Sp., MeOH	+	-	-	-	-	-	-	-	-	-	-	-
<i>C. maculata</i> , MeOH	+	+	+	+	+	-	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-
Permethrin	NA	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

EA = ethyl acetate extract; MeOH = methanol extract
 + = total kill; +/- = partial kill; - = no kill

Data Analyses and Statistics

For toxicity, the minimum concentration that provides a complete control of the mosquito larvae was defined as the MIC. The highest MIC values were chosen among the four replications of each treatment. This conservative approach should improve the accuracy of the toxicity results. For repellency, the percentage repellency from each concentration was rounded off to the nearest whole number. The median effective dosage (ED₅₀) was then calculated by Probit log concentration analysis (Finney 1971).

Results

Toxicity

All six sea lily solvent extract samples from the four species, *Himerometra magnipinna*, *Comaster multifidus*, *Comanthina* sp., and *Comatella maculata*, showed complete control at 156 ppm. Therefore, these samples were re-prepared at another serial dilution (1:1) starting at 200 ppm. Toxicity was evaluated using the MIC method. This toxicity evaluation used four replicates for each treatment, with only the highest MIC value among the replications of each treatment recorded, as listed in Table 1. The toxicity of these six sea lily samples in descending order was *C. maculata* MeOH (12.5 ppm), followed by *H. magnipinna* EA and *H. magnipinna* MeOH (25 ppm for both), *C. maculata* EA (50 ppm), *C. multifidus* MeOH (100 ppm), and *Comanthina* sp. MeOH (200 ppm).

A study of a quick bioassay for NP in 2007 included a *C. maculata* sea lily sample extracted with EA, which recorded a good insecticidal activity with MIC at 10 ppm (Chio 2007). This study evaluated the toxicity of four species of sea lily. Three of them (*H. magnipinna*, *C. multifidus*, and *C. maculata*) had been extracted either in EA or in methanol. The experiment aimed to discover whether the extracting solvents made any difference to the toxicity of the extracts against the mosquitoes. Table 2 lists their MIC values in ppm, along with the EA over MeOH ratio. Data from Table 2 show that *H. magnipinna* had an identical MIC value regardless of the solvent used in the extracting process. This happened to *C. multifidus* as well. The solvent ratio is a good tool to demonstrate the relative performance of solvents (Mitra and Brukh 2003). In this case, the EA over MeOH ratio ranged from 0.80 to 1.00, suggesting that the ethyl and methanol extracts were not significantly different. The different toxicity reported in Table 1 was most likely due to different active components in the four sea lily species.

Repellency

Table 3 lists the repellency values of six sea lily samples from the four species. The MeOH extract of *Comanthina* sp. had the

Table 2. MIC values from two solvents for three species of sea lily

Samples	MIC (PPM) in two solvents		
	EA	MeOH	EA/MeOH
<i>H. magnipinna</i>	25.00	25.00	1.00
<i>C. multifidus</i>	100.00	100.00	1.00
<i>C. maculata</i>	10.00	12.50	0.80

EA = ethyl acetate extract; MeOH = methanol extract

Table 3. Media effective dosage (ED₅₀) of sea lily extracts against *A. albopictus*

Samples	ED ₅₀ (%)	95% confidential limits
<i>H. magnipinna</i> , EA	0.38	0.15–0.62
<i>C. multifidus</i> , EA	1.25	0.94–1.66
<i>H. magnipinna</i> , MeOH	1.67	0.99–3.42
<i>C. multifidus</i> , MeOH	0.57	0.26–0.62
<i>Comanthina</i> sp., MeOH	0.32	0.22–0.60
<i>C. maculata</i> , MeOH	0.76	0.57–0.97

EA = ethyl acetate extract; MeOH = methanol extract

strongest repellency among these six samples with an ED₅₀ value of 0.32% (Table 3). The repellency (in ED₅₀) in descending order was the MeOH extract of *Comanthina* sp. (0.32%), followed by the EA extract of *H. magnipinna* (0.38%), the MeOH extract of *C. multifidus* (0.57%), the MeOH extract of *Comatella maculata* (0.76%), the EA extract of *C. multifidus* (1.25%), and the MeOH extract of *H. magnipinna* (1.67%).

Three of the four species of sea lily in this study (*H. magnipinna*, *C. multifidus*, and *C. maculata*) were extracted either in EA or in methanol (MeOH). This provides a good opportunity to compare the effects of extracting solvents as suggested by Mitra and Brukh (2003). The results of Table 4 show that the EA of *H. magnipinna* was much more potent than its methanol counterpart with the EA/MeOH ratio at 0.23.

Discussion

Few studies have been done of insect repellants derived from marine organisms. The only recent report found was from a group of Malaysian scientists (Zeti et al. 2002). They reported the repellency of seven sea squirt extracts against four species of mosquitoes including *A. albopictus* (the same species adopted in this study). Zeti

Table 4. Median effective dosage values (ED₅₀ in %) from two solvents in three species of sea lily

Samples	ED ₅₀ (%) in 2 solvents		
	EA	MeOH	EA/MeOH
<i>H. magnipinna</i>	0.38	1.67	0.23
<i>C. multifidus</i>	1.25	0.57	2.19
<i>C. maculata</i>	1.02	0.76	1.34

EA = ethyl acetate extract; MeOH = methanol extract

Table 5. Converting ED₅₀ in % to ED₅₀ in mg/cm²

Sample	ED ₅₀ (%)	ED ₅₀ (mg/cm ²)
<i>H. magnipinna</i> , EA	0.38	0.0317
<i>C. multifidus</i> , EA	1.25	0.1042
<i>H. magnipinna</i> , MeOH	1.67	0.1392
<i>C. multifidus</i> , MeOH	0.57	0.0475
<i>Comanthina</i> sp., MeOH	0.32	0.0267
<i>C. maculata</i> , MeOH	0.76	0.0633

EA = ethyl acetate extract; MeOH = methanol extract

et al. followed the ASTM standard E951-83 method, and also reported their repellency results in media effective dosage (ED₅₀), but with mg/cm² as the unit of measurement. For *A. albopictus*, their ED₅₀ values ranged from 0.0065 mg/cm² (for *Didemnum* sp.) to 0.0712 mg/cm² (for *Clavelina picta*). The extracts were found to be as potent as DEET, the positive control used in their study. However, ASTM requires human beings as bait, a walk-in growth chamber and insect counting. As some peoples are allergic to mosquito bites and the exact number of insect count is not practical for high-throughput screening operations; many entomologists are looking for alternative bioassays for insect repellents. Hence, this study developed and used simple repellency bioassay. Since the exact amount of compound (0.5 ml of known concentration) on the exact area of treatment (5 cm by 12 cm) are known in our repellency bioassay, the ED₅₀ could be converted from a percentage to mg per square center meter, as listed in Table 5. With the same unit of measurement, the best sea lily extracts in this study (*Comanthina* sp. MeOH with 0.0267 mg/cm²) had a potency of approximately a quarter of that from the best tunicate extract (0.0065 mg/cm² for *Didemnum* sp.) reported by Zeti et al. (2002).

The test of sea squirt extraction for insecticidal activities reported by Zeti et al. (2001) indicated that some tunicate species, especially the *Didemnum* sp., are highly toxic to adult *A. aegypti* with ED₅₀ values as low as 0.02 ppm. This level of toxicity against adult mosquitoes is very impressive. However, they did not test their tunicate against *A. aegypti* larvae. Tunicates are known for a wide range of biological activities (Marris 2006), while sea lilies have never been tested for any biological activities to the best of our knowledge. Although tunicates seemed to be more potent than sea lilies based on the reported data, the toxicity data for these two groups of marine organisms are not directly comparable, since they were generated by different methods, different groups, and different developmental stage of the test organisms. The potency of the crude sea lily samples was most likely underestimated. Their concentrations were calculated with the assumption that their lyophilized materials were 100% pure which is very unlikely. Therefore, their toxicity should be higher than reported here. While Zeti et al. (2001) obtained impressive results in their investigations, they

produced no follow-up report about tunicate extracts as insect control agents.

The data from the MIC method in this study matched well with the ED₅₀ values in another study of five different common mosquito larvicides (Chio 2007). An advantage of the MIC approach is that it does not require exact insect counting during the assay, and is therefore a preferred unit of measurement, especially when high volume of samples need to be tested.

Of the four species of sea lily tested, *C. maculata* and *H. magnipinna* were the most potent species against mosquito larvae in this study with a 24-hr MIC at 12.5 ppm which is considered promising (Zeti et al. 2001). These two species are worthy of further investigation.

The present results show that the repellency effect of EA and MeOH extract from sea lily is dependent on the interaction between solvent and sea lily species. The EA extract (0.38%) of the *H. magnipinna* had a higher repellency than MeOH extract (1.67%). In contrast, the repellency effect in *C. multifidus* is higher for MeOH extract (0.57%) than for the EA extract (1.25%). The EA extract of *C. maculata* was submitted to our laboratory for repellency assay with an ED₅₀ of 1.02% (Chio and Yang 2008), which seems to be less effective than its MeOH counterpart with an ED₅₀ of 0.76%.

The differential effect of varying EA/MeOH ratio for the extraction of repellency substances indicates that more active components could be extracted out from *H. magnipinna* with EA. However, in contrast, the EA/MeOH ratios for *C. multifidus* and *C. maculata* suggested that methanol might be a better solvent. These findings suggest that the existence of solvents may be species specific. Any crude extract of NP with ED₅₀ value <0.5% would be considered compromising (Zeti et al. 2002). In this case, both *Comanthina* sp. and *H. magnipinna* warrant further investigation.

The methanol or EA extracts from sea lily sampled from Taiwan showed interesting insecticidal and repellency effects against mosquito larvae and adult mosquitoes, respectively. The toxicity investigation indicates that the *H. magnipinna* and the *C. maculata* are the two most potent species among sampled sea lilies, killing all mosquito larvae at 12.5 ppm. The toxicity of these four sea lilies in descending order was *H. magnipinna*, *C. maculata*, *C. multifidus*, and *Comanthina* sp. However, these toxicity data are most likely under-reported, as the purity of test materials was assumed to be 100% which is very unlikely. With this in mind, any crude NP extracts with MIC at 12.5 ppm needs further investigation. Moreover, the EA/MeOH ratio indicates that using EA or methanol as extraction solvent has the same effect.

The results from the repellency study indicate that the EA may be a better solvent for one species (*H. magnipinna*) but the methanol (MeOH) may be a better solvent overall. The insect repellency in descending order was *Comanthina* sp. MeOH (0.32%), followed by *H. magnipinna* EA (0.38%), *C. multifidus* MeOH (0.57%), *C. maculata* MeOH (0.76%), *C. multifidus* EA (1.25%), and *H. magnipinna* MeOH (1.67%). The best repellency from the sea lily is approximately one quarter as potent as their tunicate counterpart. According to Zeti et al. (2002), any crude NP extracts that show repellency at 0.004 mg/cm² or (ED₅₀ of 0.5%) deserve a follow-up study. Therefore, both *Comanthina* sp. and *H. magnipinna* are good candidates for insecticidal and repellency effects against mosquitos.

The results from this study show that sea lilies show some interesting toxicity and repellency against mosquitoes. Further research for more species should be carried out to determine their biological activity spectrums, the residual activities in the laboratory as well as under field conditions. The active ingredients of the extracts must be isolated, purified, and identified before a patent can be filed. In

addition, knowledge of the chemical structures is needed so that their commercial potential as insecticide can be fully assessed.

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