Tunicates as a biocontrol tool for larvicides acute toxicity of Zika virus vector *Aedes aegypti*

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Abstract

In this present study, we conducted untargeted metabolic profiling using gas chromatography–mass spectrometry (GC–MS) analysis of ascidian *Didemnum bistratum* to assess the chemical constituents by searching in NIST library with promising biological properties against anti-bacterial and Zika virus vector mosquitocidal properties. Metabolites, steroids and fatty acids are abundant in crude compounds of ascidian *D. bistratum* and showed potential zone growth inhibition against bacterial strains *Kluyvera ascorbate* (10 mm). The active crude compounds of *D. bistratum* exhibited prominent larvicidal activity against the Zika vector mosquitoes of *Aedes aegypti* (LC₅₀ values of 0.44 mg/ml) and *Culex quinquefasciatus* (LC₅₀ values of 2.23 mg/ml). The findings of this study provide a first evidence of the biological properties exhibited by *D. bistratum* extracts, thus increasing the knowledge about the Zika virus vector mosquitocidal properties of ascidian. Overall, ascidian *D. bistratum* is promising and biocontrol or eco-friendly tool against *A. aegypti* and *C. quinquefasciatus* with prospective toxicity against non-target organisms.

Keywords Sea squirts · Metabolites · GC–MS · Anti-bacterial · Zika vector · Larvicidal

Introduction

Since last 50 years, Indian subcontinent has been facing mosquito infectious disease. *A. aegypti* is the major vector of chikungunya, dengue, and Zika viruses. Zika virus is transmitted to human population mainly through the bite of infected mosquitos. In urban cycle with humans as both reservoir and amplification hosts, and anthropophilic

mosquitoes as vectors (primarily, *A. aegypti* and secondarily, *A. albopictus*, *C. quinquefasciatus*). The implication of *A. aegypti* is the main vector as identified by repeated isolation of ZIKV from field-collected mosquitoes (Diallo et al. 2011), and *A. aegypti* has been suggested to be involved in transmission as ZIKV has been detected in pools of mosquitos collected in India and Brazil (Boyer et al. 2018). The matured southern house mosquitos *C. quinquefasciatus* are the primary vector of *Wuchereria bancrofti*, a nematode that causes lymphatic filariasis and also transmits *Plasmodium relictum*, a malarial parasite of birds. In May 2017, Ministry of Health and Family welfare, Government of India has reported three cases of Zika virus disease in Babunagar, Gujarat, India. The Indian Council of Medical Research screened 34,233 human samples and 12,647 mosquito samples for the presence of Zika virus (WHO 2017). This fact clearly evidences the urgent need for the implementation of innovative and more efficient vector control and discover novel drugs with therapeutic potential.

Tunicates or sea squirts are benthic fouling organisms with great ability to synthesize potential secondary metabolites with potential biomedical applications (Palanisamy et al. 2018a, b). In our previous study, we reported the

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anti-tumor activity of ascidian *Phallusia* spp. against MCF-7 breast cancer (Palanisamy et al. 2018a, b). Morris et al. (2001) reported that anthrone–anthraquinone compound, albopunctatone isolated from *Didemnum albopunctatum* collected in Australian Great Barrier Reef, showed a potential activity against malarial parasite *Plasmodium falciparum* (IC₅₀ 4.4 µM). In this study, we aimed to chemically characterize Indian Didemnid species *Didemnum bistratum* and screen their anti-Zika virus vector mosquitocidal activity and anti-bacterial activity using MIC assays.

Materials and methods

Chemicals

Methanol (MeOH, HPLC Grade) and sodium sulfate (Na₂SO₄, Sigma-Aldrich) were used as solvents for extraction of crude extract preparation and successive partition of the aqueous phase and dimethyl sulfoxide (DMSO) for larvicidal activity.

Sample collection and crude compound extraction

The colonial ascidian *D. bistratum* (Sluiter, 1905) was collected during the low tide from intertidal reef at Thoothukudi coast (Lat: 8°50'04.9"N Long: 78°15'49.7"E), Tamil Nadu, Southeast coast of India. A voucher specimen BDU/EB/SU/AS/007 has been deposited in the biomaterials lab, Department of Environmental Biotechnology, Bharathidasan University, Tamil Nadu, India. The collected samples were rinsed with sea water, fresh water to remove associated debris and salts. The collected specimen was shade dried and homogenized by mortar and pestle. The coarse powder of *D. bistratum* (weight 12 g) was extracted with methanol (150 ml) using Soxhlet apparatus and the extract was transferred into containers. Furthermore, the salt and H₂O content in crude compounds was removed using 3 g of Na₂SO₄ and filtered using filter paper size 0.41 µm. (Suarez-Jimenez et al. 2012).

GC–MS analysis

The MeOH extracts of *D. bistratum* was acquired by a newly developed GC–MS method to determine chemical composition using GC with MS Agilent Technologies GC 7890A 240 ms Ion Trap. The GC injector was set to 260 °C in split mode condition and the injector temperature was set initially 80 °C for 1 min with a 4 °C/min ramping up to 300 °C and isotherm for 300 °C for 5 min. Capillary column CP8877 VF-35 ms with 30 m × 250 µm × 0.25 µm was used. A continuous flow rate of 1 mL/min of carrier gas helium was used. The total run time was approximately 49 min. MS

detection parameters were set to initial 6 min solvent delay followed by with full scan mode 99–1000 (m/z) with the collection of data from external ionization mode mass range of 10–1000 m/z with acquisition data type—centroid, ionization control target TIC 20,000 counts, emission current 25 µA and trap temperature was set at 150 °C. The performance value of GC scan rate was at 5600 Da/s. The GC was validated with decaflorobenzophenone/isooctane [4000 MS External NCI, Pref. Eval]. Standard 1 pg/µl which is an external known standard will run regularly and track for the same RT and mass values for every run to be same. The Agilent MS/MS is controlled by an MS Workstation Version 7.0 which GLP and GMP Complied software. The workstation was loaded with the NIST Mass Spectral Search Program for NIST/EPA/NIH Mass Spectral Library Version 2.0.f. The relative metabolites from the sample were determined with the referral of m/z table of compounds with mass spectra literature reviews and mass spectral library NIST are given Table 1 (Palanisamy et al. 2018a, b).

Anti-bacterial activity

The anti-bacterial activities of ascidian *D. bistratum* extract were evaluated using disc diffusion method (Palanisamy et al. 2018a, b). In this study, there are seven bacterial pathogens of *Bacillus subtilis*: MRSA, *Streptococcus* sp., *Pseudomonas aeruginosa*, *Kluyvera ascorbata*, *Escherichia coli*, and *Klebsiella oxytoca*. All the bacterial pathogens were obtained from MTCC, Chandigarh, India. Sterile filter paper disc was used to determine anti-bacterial study of ascidian extracts, 40 µl of MeOH extracts was loaded with sterile discs and antibiotics *Ampicillin* (10 mg) used as control in this experiment. Following that the plates were swabbed with pathogenic microbes then the discs were placed with nutrient agar (Hi Media, India) plates. The inoculated plates were incubated for 24 h at 37 °C. After incubation, the zone of inhibition (mm) was measured. The results are presented as mean ± SD of the three independent values.

Mosquito culture

The field population of *A. aegypti* and *C. quinquefasciatus* (WS) larvae were collected from the densely populated area in Salem District, Tamil Nadu, India. Larvae were reared under laboratory conditions and fed with a diet of Brewer's yeast, dog biscuits (Choostix Biskies), and algae collected from ponds in a ratio of 3:2:1, respectively. The WS mosquitoes were reared till reaching the stage adult in the laboratory and were kept isolated to prevent inbreeding until identified to species. Adults were fed with wet raisins and 10% sucrose solution soaked in cotton. The adult females were deprived of sucrose from 6 h and then provided with a mouse placed in a breeding cage overnight for

Table 1 Major compounds identified from crude compounds of colonial ascidian *D. bistratum*

S. no.	RT	Area, %	Height, %	Compound	m/z
1.	17.205	1.03	1.89	Pentanoic acid	105.11
2.	18.505	0.10	0.21	Dodecanoic acid	200.31
3.	22.110	0.38	0.50	Trispiro [4.2.4.2.4.2.] heneicosane	288.51
4.	22.709	0.44	0.62	Trimethyldecalin-1-one	194.31
5.	22.957	1.46	1.19	Heptadecanoic acid	270.45
6.	24.886	0.24	0.46	1,2-Benzenedicarboxylic acid	390.55
7.	26.504	1.79	2.02	<i>Cis</i> -9-octadecene	252.48
8.	27.138	7.48	3.53	L-Ascorbyl 2,6-dipalmitate	652.95
9.	27.234	0.24	0.53	5-Eicosene	280.54
10.	27.460	0.54	0.96	Eicosanoic acid	312.53
11.	30.279	0.45	0.50	Erucic acid	338.57
12.	36.218	0.51	0.66	Myristic acid	228.37
13.	41.199	0.50	0.81	Cholesta-3,5,24-triene	366.62
14.	41.771	0.27	0.46	(22E)-Ergosta-4,7,22-trien-3-ol	396.64
15.	43.360	0.22	0.54	Cholest-5-ene,3-methoxy	368.20
16.	43.444	0.56	1.16	Stigmastan-3,5,22-trien	394.20
17.	43.513	1.34	2.33	Ergosta-5,7-dien-3-ol	396.659
18.	43.600	2.79	4.78	Tetrahydrosmilagenin	420.67
19.	44.054	58.51	46.28	Epicholesterol	215.00
20.	44.326	9.79	13.41	Cholesta-5,22-dien-3 β -ol	384.63
21.	44.789	0.46	0.92	Fucosterol	314.10
22.	44.856	4.40	5.07	Allocholesterol	386.66
23.	45.971	0.57	0.78	Chondrillasterol	412.70
24.	46.244	0.26	0.70	Propionic acid	74.079
25.	47.131	0.27	0.45	3-Ethenylcholestan-3-ol	414.71
26.	47.299	0.15	0.26	Cholestan-5-ol-6-one	402.65
27.	47.369	0.41	0.78	Hydroquinone	110.11
28.	47.650	0.85	1.70	1,4-Cyclohexadiene	80.13
29.	47.785	0.52	1.02	11 β -18-Epoxy lanostane	428.74

RT retention time

blood feeding. Adult mosquitoes were maintained under the same environmental conditions as the larvae (Arivoli and Samuel 2011).

Larval mortality assay

Bioassays were performed according to the method recommended by World Health Organization and as suggested by Elumalai et al. (2017). Twenty-five fourth instar larvae were marked in 249 mL of distilled water with 1 mL DMSO as negative control. After 24 h of exposure, dead larvae were calculated and the proportion of dead larvae was calculated from the average of three replicates. Larval mortality was corrected using Abbott's formula. The percentage average mortality of three replicates was used to calculate lethal concentration (LC₅₀ and LC₉₀) by prohibit analysis.

Results and discussion

Metabolites from *Didemnid* species

The GC–MS analysis revealed the presence of > 30 metabolites from the MeOH extract of *D. bistratum* (Fig. 1). The GC–MS chromatogram shows the peak area separation of the components and major components epicholesterol (58.51%), dinorcholesta-5,22-dien-3-ol (9.79%), 1-(+)-ascorbic acid 2,6-dihexadecanoate (7.48%), allocholesterol (4.40%) and other minor compounds of tetrahydrosmilagenin (2.79), 9-octadecene (1.79%), oxirane (1.52%), ergosta-5,7-dien-3-ol (1.34%), heptadecanoic acid (1.46%), pentanoic acid (1.03%), 1,4-cyclohexadiene (0.85%), 5-eicosene (0.24%) (Table 1). Similarly, compound of 5-eicosene was previously reported from ascidians *Phallusia* sp. with potential cytotoxicity against colon

Fig. 1 GC chromatogram for the MeOH extract of colonial ascidian *D. bistratum*

cancer cells (Palanisamy et al. 2018a, b) and metabolite, saturated fatty acid eicosanoic acid from *Didemnum psammathodes* (Kumaran and Bragadeeswaran 2014). Similar to ascidian chemical constituents, sterols are abundance in sponges and marine algae were reported by Santalova et al. (2004), Zhen et al. (2015). Avis and Bélanger (2001) reported that heptadecanoic acid promises the anti-microbial activity against fungal pathogens. The identified compounds from MeOH extract of *D. bistratum* demonstrate the presence of vital compounds which possess the reported biological activity. This study revealed that the stronger extraction capacity of MeOH could have produced number of active constituents with unprecedented structures responsible for many biomedical applications.

Anti-bacterial and larvicides acute toxicity of Zika virus vector *A. aegypti*

The biological activity of *D. bistratum* was screened against anti-Zika vector properties, the results are given in Table 2. We also tested anti-microbial activity against seven bacterial human pathogens (Table 3). The MeOH extract of *D. bistratum* exhibited potential zone growth inhibition against human pathogenic strain MSRA (9.66 ± 0.57 mm), *Klebsiella oxytoca* (9.33 ± 0.57 mm) and minimum zone was measured with *E. coli* (1.33 ± 0.57 mm) at concentration 25 mg/ml. Hussain and Ananthan (2009) reported that crude methanol extract of *D. psammathodes* showed potential zone growth inhibition against *Salmonella typhii* (15 mm) and *D.*

Table 2 Anti-bacterial activity MeOH extract of *D. bistratum* against the bacterial pathogens

S. no.	Bacterial pathogens	Control (mm)	<i>Didemnum bistratum</i> (mm)
1	<i>Bacillus subtilis</i>	8.8 ± 0.03	8.53 ± 0.03
2	MRSA	9.1 ± 0.1	9.66 ± 0.57
3	<i>Streptococcus</i> sp.	8.23 ± 0.06	4.56 ± 0.06
4	<i>Pseudomonas aeruginosa</i>	9.43 ± 0.05	8.66 ± 0.57
5	<i>Kluyvera ascorbata</i>	10.6 ± 0.1	9.00 ± 0.00
6	<i>Escherichia coli</i>	9.93 ± 0.15	1.33 ± 0.57
7	<i>Klebsiella oxytoca</i>	8.56 ± 0.11	9.33 ± 0.57

candidum showed poor growth inhibition against *Staphylococcus aureus* (1 mm) at 20 μ l concentration. Compared to the results of our study, Selva Prabhu et al. 2012 reported maximum zone growth inhibition against *E. coli* (10 mm) of ascidian *Polyclinum madrasensis* collected from Tuticorin Harbor area. Palanisamy et al. (2016) reported significant anti-microbial activity against *Pseudomonas* sp compared to control drug ($p < 0.01$) of invasive ascidian *Styela plicata* from Messina, Italy. He also documented the poor zone growth against the strain *Staphylococcus aureus*.

The different dose concentrations (0.02–1.5 mg/l) were chosen to test against second to fourth instar larvae of *A. aegypti* and *C. quinquefasciatus*. The promising mosquito larvicides acute toxicity of Zika virus vector was observed against larval stages of *A. aegypti* with values of LC50

Table 3 Larvicidal activity of ascidian *D. bistratum* against different instar larvae of dengue vector *Ae. aegypti* and *Cx. quinquefasciatus*

Mosquito vector	No. of larvae	LC ₅₀ (LCL–UCL) mg/ml	LC ₉₀ (LCL–UCL) mg/ml	χ^2	df
<i>A. aegypti</i>					
IInd instar	375	0.44 (0.16–2.18)	21.04 (18.67–25.87)	1.56	3
IIIrd instar	375	1.34 (1.21–2.49)	18.56 (15.56–25.87)	3.54	3
IVth instar	375	1.98 (0.98–2.89)	25.95 (22.0–30.91)	4.009	3
<i>C. quinquefasciatus</i>					
IInd instar	375	2.23 (1.94–2.32)	6.73 (5.90–8.45)	1.98	3
IIIrd instar	375	2.95 (2.73–3.09)	8.03 (7.90–9.67)	2.23	3
IVth instar	375	4.23 (3.42–6.83)	9.71 (7.73–10.83)	2.53	3

LC₅₀ lethal concentration 50% mortality, LC₉₀ lethal concentration 90% mortality, LCL 95% lower confidence limits, UCL 95% upper confidence limits, χ^2 Chi-square, df degrees of freedom

and LC90 0.4436 mg/ml; 21.047 mg/ml (IInd instar); 1.34167 mg/ml; 18.5678 mg/ml (IIIrd instar) and 1.9856 mg/ml; 25.9560 mg/ml (IVth instar) and *Cx. quinquefasciatus* of LC50 and LC90 values of 2.2371; 6.7321 mg/ml (IInd instar); 2.9561; 8.0317 mg/ml (IIIrd instar) and 4.239007; 9.71364 mg/ml (IVth instar), respectively. The previous studies of secondary metabolites from the water and butanol extracts of the ascidians *Ascidia sydneinsis*, *Microcosmus goanus* and *Phallusia nigra* of the Cuban coast have been reported with inhibition (50%) against the malarial protozoan *P. falciparum* at concentrations of 20.9, 17.5 and 29.4 µg/ml (Mendiola et al. 2006). Compound, Mollamide B isolated from the ascidian *D. molle* showed promising anti-plasmodial activity against the protozoan larva of *P. falciparum* with IC50 of 2.0 µg/ml (Donia et al. 2008). Two indole spermidine alkaloids, Didemnidines A, B reported Didemnum sp collected from Tiwai Point, New Zealand and Didemnidine B has showed modest in vitro growth inhibition against *P. falciparum* and *T. brucei* rhodesiense IC50 value of 8.4 and 9.9 µM (Finlayson et al. 2011). In recent study, Mahyoub et al. (2017) reported that the sea-grass *Halodule uninervis* exhibited significant acute toxicity against fourth instar larvae dengue vector *A. aegypti* (LC50 295.62 ppm). The crude compounds of ascidian *Didemnum* sp. have strong potential activity against the anti-Zika vector mosquitocidal acute toxicity. The further research on ascidian natural product diversity was conducted to find drug candidates against malarial larvicidal to prevent the infectious disease. The obtained results could serve as a baseline for further research towards designing active anti-bacterial agents against human pathogens and Zika vector mosquito larvicidal to prevent infectious disease.

Conclusions

The results of this study demonstrated the chemical constituents of least studied Indian ascidian fauna in habituating the area of Gulf of Mannar Biosphere and demonstrated the

biological activity of ascidian of *Didemnum* sp. The extract of *D. bistratum* showed promising anti-Zika vector mosquito larvicidal action than anti-microbial activity. The outcome of this study revealed that ascidian *D. bistratum* is promising candidate due to the presence of anthroquinone and indole spermidine alkaloids group of compounds responsible for the biocontrol or eco-friendly tool against *A. aegypti* and *C. quinquefasciatus* with potential toxicity against non-target organisms. The results of this study open new hope for discovering new drug candidate from Indian ascidian with potential therapeutic in the treatment of mosquito-borne diseases and bacterial infections.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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