 doi:10.20944/preprints201810.0113.v1

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

Velusamy Arumugam[1], Manigandan Venkatesan[2], Karthi Sengodan[3], Umamaheswari Sundaresan[1]*, Satheesh Kumar Palanisamy[4]

[1]*Department of Environmental Biotechnology, School of Environmental Sciences, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India.*
[2]*Department of Medical Biotechnology, Chettinad Academy of Research and Education, Kelambakkam, Chennai – 603 013, Tamil Nadu, India.*
[3]*Department of Biochemistry, K.S. Rangasamy College of Arts and Science (Autonomous), Tiruchengode–637 215, Namakkal, Tamil Nadu, India.*
[4]*Ryan Institute, School of Natural Science, Department of Zoology, National University of Ireland, Galway, Ireland.*

*Corresponding author: myscienceworld@gmail.com

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* and *Cluex quinquefasciatus* (LC_{50} values of 0.4436 to 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* are promising and biocontrol or eco-friendly tool against *A. aegypti* and *C. quinquefasciatus* with prospective toxicity against non-target organisms.

Key words: Sea Squirts, Metabolites, GC-MS, Anti-bacterial, Zika vector**,** Larvicidal

1. Introduction

Since last 50 years, Indian subcontinent have been facing mosquito infectious disease. *Aedes 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

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 October 2018 doi:10.20944/preprints201810.0113.v1

reservoir and amplification hosts, and anthropophilic mosquitoes as vectors (primarily, *Aedes aegypti* and secondarily, *Ae. albopictus*, *Cluex quinquefasciatus*). The implication *of Ae. aegypti* is the main vector as identified by repeated isolation of ZIKV from field-collected mosquitoes (Diallo et al. 2011), and *Ae. 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* is the primary vector of *Wuchereria bancrofti*, a nematode that causes lymphatic filariasis and also transmit also transmits *Plasmodium relictum*, a malarial parasite of birds. In May 2017, Ministry of Health and Family welfare, Govt. of India has reported three cases of Zika virus disease in Babunagar, Gujarat, India. The Indian Council of Medical Research was 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. 2018). In our previous study, we reported the anti-tumor activity of ascidian Phallusia spp. against MCF7 breast cancer. Morris et al. 2011 reported anthrone-anthraquinone compound, albopunctatone isolated from *Didemnum albopunctatum* collected in Australian Great Barrier Reef, showed a potential activity against malarial parasite Plasmodium falciparum (IC_{50} 4.4 µM). In this present study, we aimed to chemical characterization of India Didemnid species *Didemnum bistratum* and screening their Anti-Zika virus vector mosquitocidal activity and anti-bacterial activity using MIC assays.

2. Materials and methods

2.1 Chemicals

MeOH- methanol (HPLC Grade), Na_2SO_4 -Sodium sulphate (Sigma-Aldrich) was used solvents for extraction of crude extracts preparation and successive partition of the aqueous phase and Dimethyl sulfoxide (DMSO) for Larvicidal activity.

2.2 Sample collection and preparation of MeOH extracts

The colonial ascidian *Didemnum 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

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 October 2018 doi:10.20944/preprints201810.0113.v1

powder was extracted with methanol using Soxhlet apparatus and the extract was transferred into containers. Further, the extract was treated to remove salt content and water using standard protocol (Suarez-Jimenez et al. 2012) and concentrated using rotary evaporator under reduced pressure.

2.3 GC-MS analysis

The MeOH extracts of ascidians were acquired by a newly developed GC-MS method for analyses their chemical composition by GC with MS Agilent Technologies GC 7890 C 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-35ms with 30m x 250 µm x 0.25 µm were used. A continuous flow rate of 1mL/min of carrier gas Helium was used. The total run time was approximately 60 min. MS detection parameters were set to initial 3 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 20000 counts, emission current 25µAmps and trap temperature was set at 150°C. The performance value of GC Scan rate at 5600 Da/s. The GC was validated with Decaflorobenzophenone/Isooctane [4000 MS External NCI, Pref. Eval. Standard 1pg/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 *mz* table of compounds with mass spectra literature reviews and mass spectral library NIST are given table 1(Palanisamy et al. 2018).

2.4 Anti-bacterial activity

The antibacterial activities of *D. bistratum* MeOH extract was evaluated using the disc diffusion method (Palanisamy et al. 2018). The seven bacterial pathogens of *Bacillus subtilis, MRSA, Streptococcus* sp, *Pseudomonas aeruginosa, Kluyvera ascorbata, Escherichia coli, and Klebsiella oxytoca* were tested in this study. Sterile filter paper disc was used in conducting this anti-bacterial study. 40 µl of MeOH extract sample was loaded with sterile discs and *Ampicillin* antibiotics (10 mg) used as controls. Later 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 hrs at 37°C. After incubation, the zone of inhibition (mm) was measured. The results are presented as means ±SD of the three independent values.

2.5 Mosquito Culture

The field population of *Ae. aegypti* and *Cx. 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

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 October 2018 doi:10.20944/preprints201810.0113.v1

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 blood feeding. Adult mosquitoes were maintained under the same environmental conditions as the larvae.

2.6 Larval mortality assay

Bioassays were performed according to the method recommended World Health Organization and as suggested by Elumalai et al. 2015. Twenty-five fourth instar larvae were marked in 249 mL of distilled water with 1 mL DMSO as negative control. After 24 hours of exposure, dead larvae were calculated and the proportion of dead larvae 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 (LC_{50} and LC_{90}) by prohibit analysis.

3. Results and Discussion

3.1 Metabolites from Didemnid species

The GC-MS analysis revealed the presence of >30 metabolites from the MeOH extract of *D. bistratum* (Figure 1). The GC-MS chromatogram shows the peak area separation of the components and major components Epicholestanol (58.51%), Dinorcholesta-5, 22-dien-3-ol (9.79 %), l-(+)-Ascorbic acid 2, 6-dihexadecanoate (7.48%), Allocholesterol (4.40%) and other minor compounds of Etrahydrosmilagenin (2.79), 9-Octadecene (1.7 9%), 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 cancer cells (Palanisamy et al. 2018) 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 (Santalova et al. 2004; Zhen et al. 2015). Avis and Bélanger (2001) was reported that Heptadecanoic acid promising the anti-microbial activity against fungal pathogens (Avis and Bélanger, 2001). The identified compounds from the MeOH extract of *D. bistratum* demonstrate the presence of vital compounds which possess the reported biological activity. The investigation concluded that the stronger extraction capacity of MeOH could have been produced number of active constituents with unprecedented structures responsible for many biomedical applications.

3.2 Anti-bacterial and larvicides acute toxicity of Zika virus vector Aedes aegypti

The biological activity of *D. bistratum* was screened against Anti-Zika vector properties, the results are given in Table 3. We also tested Anti-microbial activity against 7 bacterial human

pathogens (Table 2). 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 ± 0.57mm) and minimum zone was measured with *E. coli* (1.33±0.57 mm) at concentration 25mg/ml. Hussain and Ananthan, 2009 reported crude methanol extract of *D. psammathodes* showed potential zone growth inhibition against *Salmonella typhii* (15 mm) and *D. candidum* showed poor growth inhibition against *Staphylococcus aureas* (1 mm) at 20 µl concentration. Compare 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 compare 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 to 1.5 mg/L) were chosen to test against second to fourth instar larvae of *Ae. Aegypti and Cx. quinquefasciatus*. The promising mosquito larvicides acute toxicity of Zika virus vector was observed against larval stages of *A. aegypti* with values of LC_{50} and LC_{90} 0.4436 mg/ml; 21.047 mg/ml (II^{nd} instar); 1.34167 mg/ml; 18.5678 mg/ml (III^{rd} instar) and 1.9856 mg/ml; 25.9560 mg/ml (IV^{th} instar) and *Cx. quinquefasciatus* of LC_{50} and LC_{90} values of 2.2371; 6.7321 mg/ml (II^{nd} instar); 2.9561; 8.0317 mg/ml (III^{rd} instar) and 4.239007; 9.71364 mg/ml (IV^{th} instar) respectively (Table 3). 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; Palanisamy et al. 2017) . Compound, Mollamide B was isolated from the ascidian *D. molle* showed promising Anti-plasmodiadial activity against the protozoan larva of *P. falciparum* with IC_{50} of 2.0 µg/ml (Donia et al. 2008). The didemnum sp. of two indole spermidine alkaloids, didemnidines A, B reported from Tiwai Point, New Zealand and Didemnidine B has showed modest in vitro growth inhibition against P. falciparum and T. brucei rhodesiense IC_{50} value of 8.4 and 9.9 µM (Finlayson et al. 2011). In recent study, Mahyoub et al. 2017 reported the Seagrass Halodule uninervis exhibited significant acute toxicity against 4^{th} instar larvae dengue vector *Ae. aegypti* (LC_{50} 295.62 ppm). The crude compounds of ascidian *Didemnum* sp has strong potential activity against the Anti- Zika vector mosquitocidal acute toxicity. The further research on ascidian natural product diversity to find drug candidates against malarial larvicidal to prevent the infectious disease. The obtained results could serve as a basis for further research towards design active anti-bacterial agents against human pathogens and Zika vector mosquito larvicidal to prevent infectious disease.

4. Conclusions

The results of this study demonstrated the chemical constituents of least studied Indian ascidian fauna in habitating the area of Gulf of Mannar Biosphere and demonstrated the biological activity of ascidian of *Didemnum* sp. The extract of *D. bistratum* seems to more Anti-Zika vector mosquito larvicidal action than anti-microbial action. It can be concluded that ascidian *D. bistratum* are promising due to the presence of anthroquinone and indole spermidine alkaloids compounds and could be used as a biocontrol or eco-friendly tool against *A. aegypti* and *C. quinquefasciatus* with prospective toxicity against non-target organisms. In further research, bioassay guided fractionation and identification of active substances from the Didemnum sp. against Zika-vector mosquitos *Aedes aegypti* and *Cluex quinquefasciatus* and human pathogens.

Conflict of Interest

All the authors declare that no conflict of interest.

Acknowledgement

This work is supported by the DST-Science and Research Engineering Board, India under grant SB/YS/LS-374/2013, UGC-FIST and UGC-SAP, New Delhi.

References

Avis TJ, Bélanger RR (2001) Specificity and mode of action of the antifungal fatty acid cis-9-heptadecenoic acid produced by Pseudozyma flocculosa. Appl Environ Microbiol. 67: 956-960.

Boyer S, Calvez E, Chouin-Carneiro T, Diallo D, Failloux AB (2018) An overview of mosquito vectors of Zika virus. Microbes and infection. Microbes and Infection. xxx 1-15. https://doi.org/10. 1016/ j.micinf.2018.01.006.

Donia MS, Wang B, Dunbar DC, Desai PV, Patny A, Avery M, Hamann MT (2008) Mollamides B and C, cyclic hexapeptides from the Indonesian tunicate *Didemnum molle*. J Nat Prod 71(6): 941-5.

Diallo D, Sall AA, Diagne CT, Faye O, Faye O, Ba Y, Hanley KA, Buenemann M, Weaver SC, Diallo M (2011) Zika virus emergence in mosquitoes in southeastern Senegal. PloS one 9(10): e109442.

Elumalai D, Hemalatha P, Kaleena PK (2017) Larvicidal activity and GC–MS analysis of Leucas aspera against Aedes aegypti Anopheles stephensi and Culex quinquefasciatus. J Sau Soc Agricult Sci 16: 306-313.

Finlayson R, Pearce AN, Page MJ, Kaiser M, Bourguet-Kondracki ML, Harper JL, Webb VL, Copp BR (2011) Didemnidines A and B, indole spermidine alkaloids from the New Zealand ascidian Didemnum sp. Journal of natural products 74(4):888-92.

Hussain SM, Ananthan G (2009) Antimicrobial activity of the crude extracts of compound ascidians, Didemnum candidum and Didemnum psammathodes (Tunicata: Didemnidae) from Mandapam (South East Coast of India). Curr Res J Biol Sci 1(3):168-71.

Kumaran NS, Bragadeeswaran S (2014) Nutritional Composition of the Colonial Ascidian Eudistoma viride and Didemnum psammathodes. Biosci Biotech Res Asia 1:331-338**.**

Mahyoub JA, Panneerselvam C, Murugan K, Roni M, Trivedi S, Nicoletti M, Hawas UW, Shaher FM, Bamakhrama MA, Canale A, Benelli G (2017) Seagrasses as sources of mosquito nano-larvicides? Toxicity and uptake of Halodule uninervis-biofabricated silver nanoparticles in dengue and Zika virus vector Aedes aegypti. Journal of Cluster Science 28(1):565-80.

Mendiola J, Hernandez H, Sariego I, Mendiola J, Hernández H, Sariego I, Rojas L, Otero A, Ramírez A, de Los Angeles Chavez M, Payrol JA, Hernández A (2006) Antimalarial activity from three ascidians: An exploration of different marine invertebrate phyla. Trans R Soc Trop Med Hyg 100: 909–916**.**

Morris LA, Jaspars M, Kettenes-van den Bosch JJ, Versluis K, Heck AJ, Kelly SM, Price NC (2001). Metal binding of *Lissoclinum patella* metabolites. Part 1: Patellamides A, C and ulithiacyclamide A. Tetrahedron 57(15):3185-97.

Palanisamy SK, Arumugam V, Peter MD, Sundaresan U (2018) Patterns of chemical diversity in the marine ascidian Phallusia spp.: anti-tumor activity and metabolic pathway inhibiting steroid biosynthesis. 3 Biotech 8(5):251.

Palanisamy SK, Morabito R, Remigante A, Spanò N, La Spada G, Giacobbe S, Marino A (2016) Biological activity of extract from Styela plicata and Ascidia mentula (Ascidiacea). Journal of Biological Research-Bollettino della Società Italiana di Biologia Sperimentale 27: 89(1).

Palanisamy SK, Rajendran NM, Marino A (2017) Natural products diversity of marine ascidians (tunicates; ascidiacea) and successful drugs in clinical development. Nat Prod Bioprospect 7(1)1-111.

Palanisamy SK, Arumugam V, Rajendran S, Ramadoss AS, Nachimuthu S, Peter MD, Sundaresan U, 2018. Chemical diversity and antiproliferative of marine algae. J Natural Product Research. DOI:10.1080/14786419.2018.148870.

Santalova EA, Makarieva TN, Gorshkova IA, Dmitrenok AS, Krasokhin VB, Stonik VA (2004) Sterols from six marine sponges. Biochem Syst Ecol 32(2):153-167.

 doi:10.20944/preprints201810.0113.v1

Selva Prabhu A, Ananthan G, Sathish Kumar R (2012) Antibacterial activity of marine ascidian *Polyclinum madrasensis* (Sebastian, 1952) against human clinical isolates. Int J Ins Phar Life Sci 2(3).

Suarez-Jimenez GM, Burgos-Hernandez A, Ezquerra-Brauer JM (2012) bioactive peptides and depsipeptides with anticancer potential: Sources from marine animals. Mar dru 10(5):963-86.

WHO (2017) Zika virus infection - India. http://www.who.int/csr/don/26-may-2017-zika-ind/en/.

Zhen XH, Quan YC, Jiang HY, Wen ZS, Qu YL, Guan LP (2015) Fucosterol, a sterol extracted from Sargassum fusiforme, shows antidepressant and anticonvulsant effects. Eur J Pharmacol 768:131-8.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 October 2018 doi:10.20944/preprints201810.0113.v1

Table 1. Major compounds identified in the MeOH extract of *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	Cis-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	Epicholestanol	215.00
20.	44.326	9.79	13.41	Cholesta-5,22-dien-3β-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-Epoxylanostane	428.74

**RT-Retention Time*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 October 2018 doi:10.20944/preprints201810.0113.v1

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

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

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**
***Ae. aegypti*)**			
IInd instar	375	0.44 (0.16-2.18)	21.04 (18.67-25.87)
IIIrd instar	375	1.34 (1.21-2.49)	18.56 (15.56-25.87)
IVth instar	375	1.98 (0.98-2.89)	25.95 (22.00-30.91)
Cx. quinquefasciatus			
IInd instar	375	2.23 (1.94-2.32)	6.73 (5.90-8.45)
IIIrd instar	375	2.95 (2.73-3.09)	8.03 (7.90-9.67)
IVth instar	375	4.23 (3.42-6.83)	9.71 (7.73-10.83)

LC_{50} Lethal concentration 50% mortality, LC_{90} Lethal concentration 90% mortality, LCL 95% lower confidence limits, UCL 95% upper confidence limits, $\chi 2$ chi square, df degrees of freedom.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 October 2018 doi:10.20944/preprints201810.0113.v1

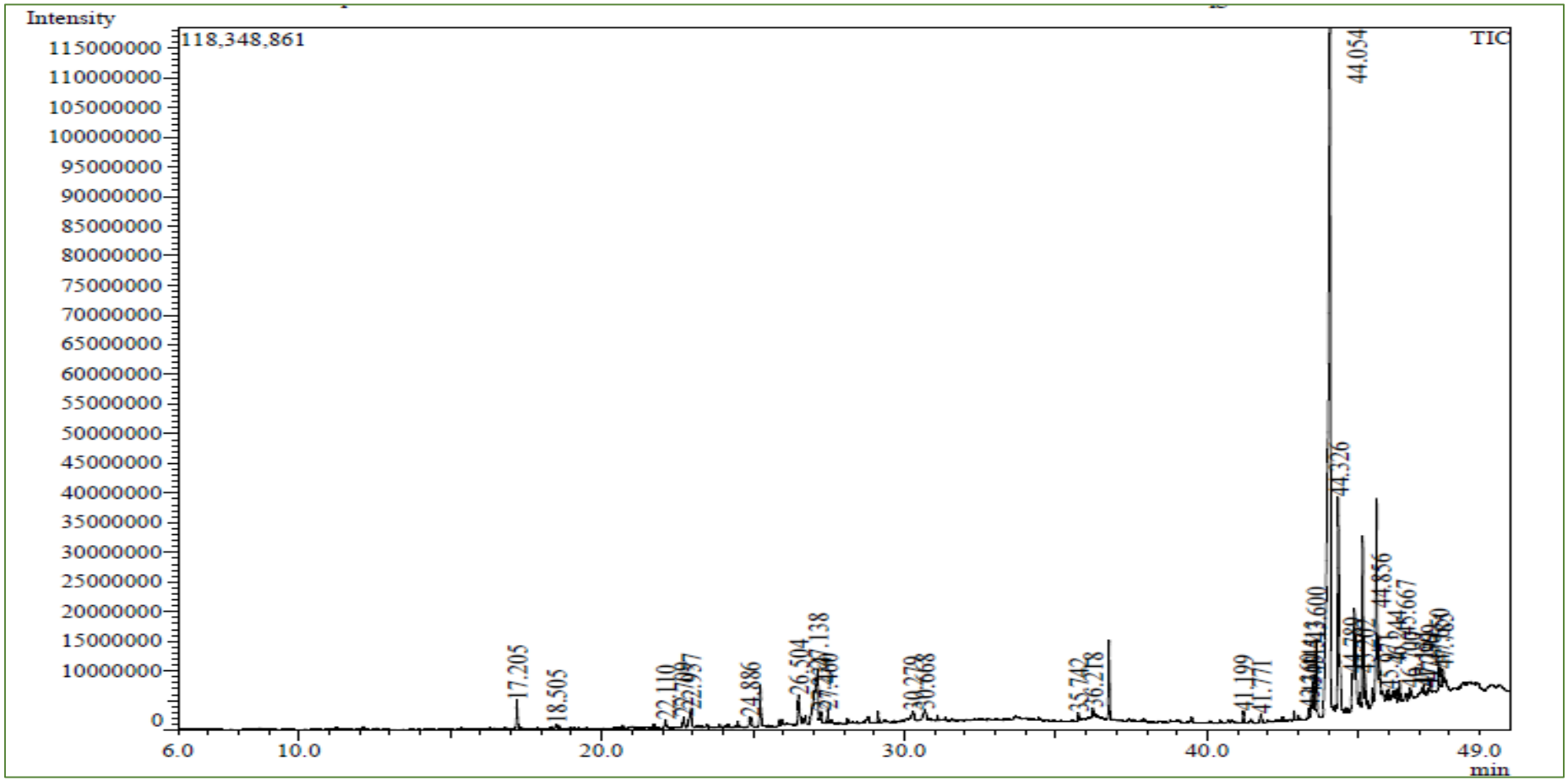

Figure 1. GC chromatogram for the MeOH extract of *D. bistratum*