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Mosquito larvicidal and bactericidal action of crude extract of marine brown alga *Turbinaria conoides*

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Abstract

Mosquito related problems and bacterial resistance against synthetic chemicals have become acute in recent years, therefore to solve this, the search for biochemicals is on. Marine brown algae have been traditionally used as nutraceuticals and it contains a rich source of bioactive compounds, hitherto many novel bioactive compounds are yet to be explored. For this research, a marine brown alga of *Turbinaria conoides* was collected from Mandapam, Tamil Nadu and compounds were extracted using various solvents such as polar (ethanol, acetone) and nonpolar (benzene and diethyl ether). The crude extracts were checked for mosquito larvicidal (*Culex quinquifasciatus*), bactericidal (*Staphylococcus aureus*) and antifungal (*Aspergillus niger*) activity. The crude extract of diethyl ether showed potential larvicidal activity as 50% lethal concentration (LC_{50}) was noted at 104 mg/L followed by acetone (469 mg/L), benzene (781 mg/L) and ethanol (1917 mg/L), respectively. Also, an active antibacterial effect in diethyl ether extract was superior as a 4 mm zone of inhibition (ZoI) was found with 0.1 mg extract followed by acetone (4 mm ZoI at 1.2 mg extract). Intriguingly, no fungicidal was observed in any extract which rather supported the fungal growth. Based on this result, the acetone and diethyl ether extract showed worthy duel activity compounds and analyzed for GC-MS and observed three novel compounds in the acetone extract. These compounds might be involved in the antilarval and antibacterial action and will be analyzed in the future. The results of this study are very useful for our human society because of the potential for a better life through the treatment of diseases.

Keywords: Bactericide, Brown algae, Mosquito larvicide, Phenolic compound, *Turbinaria conoides*

1. Introduction

Marine macroalgae, especially brown algae, have important ecological and commercial value to many regions of the world because they are valuable food and feed resources, as well as having pharmaceutical value [1]. It is rich in vitamins, minerals, proteins, steroids, carotenoids, terpenoids, xanthophylls, chlorophyll, saturated and unsaturated fatty acids, amino acids, antioxidants (polyphenols, alkaloids, halogenated compounds), polysaccharides (agar, carrageenan, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol, and fucoidan) and dietary fibers [1,2]. Since as early as 3000 Before Crist (BC), algal were considered important as traditional remedies and their use has significantly expanded in the past three decades due to the global reality of

bacterial resistance to existing antibiotics [3] and insect resistance to insecticides [4]. Brown algae metabolites probably have diverse simultaneous functions in them and can act as allelopathic, antimicrobial, antifouling and antilarval agents [5]. They are also used by the pharmaceutical industry in drug development to treat bacterial and fungal diseases and various illnesses, and even in the treatment of acquired immune deficiency syndrome (AIDS) [2]. Sessile algae such as brown algal seaweeds are more prone to bacterial biofilm formation and consumption by invertebrates and its larvae compared to motile algae. Allelochemicals have been identified in several sessile brown algal species which aid algae to combat with other organisms for anchoring space and survival. Many of these compounds are toxic to other organisms and are currently being screened for antibacterial and antilarval potential [6-8]. Presently algae represent about 9%of biomedical compounds obtained from the sea. The harnessing and bioengineering of recently characterized allelochemicals represent a potential area of new marine algal antibacterial [2] and antilarval agents [6,9]. The mosquito is a primary vector for prevalent deadly diseases such as malaria, dengue, yellow fever, West Nile Virus, filariasis, and encephalitis. It can also transmit several other illnesses that can present serious health problems to human beings [10,11]. Moreover, frequent use of synthetic insecticides leads to a destabilization of the ecosystem and enhanced resistance in pests, suggesting a need for alternatives [4]. Marine macroalgal extracts may be the rich alternative sources of mosquito larvicidal agents which are biodegradable, nontoxic products and potentially suitable for use in the control of mosquito larvae. Over the last few decades, studies on marine brown algal extract against mosquito larvae have been conducted around the world [6,8,12] and its synergistic effects with antimicrobials also investigated. *T. conoides* is a marine brown alga which contains many secondary metabolites, however there are not many research findings on mosquito larvicidal and antimicrobial properties. Therefore, the search for new bioactive compounds for the above-mentioned crisis, consequently collected *T. conoides* and were extracted using various solvents (polar and nonpolar) and analyzed for mosquito larvicidal, antibacterial and antifungal action. This research is improving the knowledge about the usefulness of these widely available, easy to obtain, and cost effective natural extracts against human pathogens and mosquito-borne diseases.

2. Materials and methods

2.1 Sample collection and processing

The marine brown algal seaweed of *T. conoides* was collected by deep-sea divers at the shore of Mandapam, nearby Rameshwaram, Gulf of Mannar, Tamil Nadu, and India. The collected samples were thoroughly washed in running tap water and air-dried at room temperature in the shade for 7 d**,** then powdered using a mixer grinder and stored in an airtight container. It was very tough for the grinding of this alga due to its stiffness after the drying process.

2.2 Organic solvent extraction

Four different organic solvents (ethanol and acetone as polar, benzene and diethyl ether as non-polar) were used to prepare the algal extract. Ten grams of *T. conoides* powder was mixed with 100 ml of each solvent separately and kept in an orbital shaker at 120 rpm for 24 h at room temperature. Then the extract was filtered using a Whatman 74 No.1 filter paper or centrifuged at 5000 rpm for 10 min and transferred to a Petri dish and allowed to evaporate at 40-75 ˚C overnight and weighed. Finally, the dried extract was dissolved in 1.5 ml of respective solvents.

2.3 Mosquito larvicidal assay

Mosquito larvae (*C. quinquifasciatus*) were collected from the stagnant water from the nearby area of Pallavaram, Chennai, and Tamil Nadu**.** Larvae were maintained at 27±2 ˚C and were fed with dog biscuits and glucose 79 in a 5:100 ratio. Well plate (24 well) was used and added 2 ml of sterile tap water with 50 µl of each extract and 5 larvae were transferred into each well using a thin brush. A total of 20 larvae were added in 4 wells for each treatment. The experiment was performed three times. 50 µl of each solvent alone was added in the well as a control. The larval mortality was calculated after 24 h of the exposure period. The mortality rate of the larvae was calculated by

$$Percentage\ mortality\ of\ larvae = \frac{No.\ of\ dead\ larvae}{No.\ of\ larvae\ introduced} X\ 100$$

2.4 Antimicrobial activity

The different solvent extracts of the algae were subjected to antibacterial assay by Kirby Bauer disc diffusion method using Muller Hinton agar plates against the human pathogen of *S. aureus*. The log phase bacterial 88 culture was maintained in LB broth at 37 ˚C. The different volume (10, 20, 40 µl) of crude extract disc was prepared 89 using sterile Whatman No. 1 filter paper (6 mm diameter) by adding and drying method (5 µl every time). For positive 90 control, 10 mg chloramphenicol disc was used (Himedia), for the negative control 40 µl respective solvent alone was sed in the disc. The 50 µl of 12 h old *S. aureus* was spread on Muller Hinton agar plates and placed extract disc and control disc and incubated for 24 h at 37 ˚C, and measured zone of inhibition (ZoI) as in diameter. Whereas antifungal activity, the media inclusion method using a potato dextrose agar (PDA) for fungal pathogens *A. niger*. The PDA plates were prepared with 500 µl of different extract separately and inoculated the *A. niger* at the center of the plate and incubated at 25 ˚C for a week and observed the growth and the PDA plate without extract as a positive control.

2.5 GC-MS analysis of the extract

The acetone extract was chosen for compound analysis in GC-MS, due to its duel activity (larvicidal and bactericidal). The extract was analyzed in Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in the thickness of film) at Tamil Nadu Veterinary University, Chennai. A volume of 1 µl sample extract was injected manually and kept the temperature at 230 ˚C and 220 ˚C for injection port and detector, respectively. The column temperature was at 100 ˚C for 5 min initially and then increased at 10 ˚C/min to 250 ˚C. The mass spectrum was set with electron impact ionization mode at 1500 V with source temperature of 240 ˚C and quad temperature at 150 ˚C. The mass spectral acquisition was performed using GC-MSD Agilent Chem Station Software. The structural determination was by comparison of mass spectral patterns to the National Institute of Standards and Technology (NIST) library.

3. Results

3.1 Mosquito larvicidal assay

The results of mosquito larvicidal activity of four different solvent extracts (acetone, benzene, ethanol, and diethyl ether) of *T. conoides* against *C. quinquefasciatus* larvae were analyzed and compared with previously studies. (Figure 1 and Table 1). The maximum of 80% mortality was observed in acetone and benzene extract in 24 h of incubation, whereas ethanol extract and diethyl ether extract shown 60% and 40% in 50 µl crude extract respectively. However, in the 50 µl of crude extract of these solvent showed different larvicidal activities due to its different concentration of the extracted compound in the extract such as 2300 mg/L in ethanol extracts, followed by 1250 mg/L in benzene, 750 mg/L in acetone and the least of 83 mg/L in diethyl ether extract. The ethanol extract showed the highest efficiency of extract concentration however the less larvicidal activity as observed LC_{50} of 1917 mg/L, whereas diethyl ether extract revealed higher efficiency of LC_{50} of 104 mg/L followed by 469 mg/L for acetone and 781 mg/L for benzene extract, respectively.

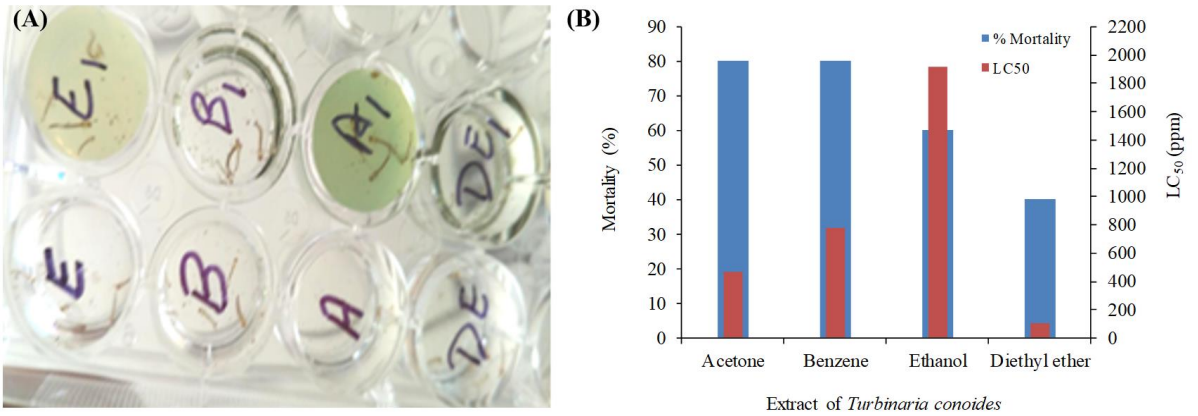

Figure 1 Mosquito larvicidal activity of different solvent extracts of marine brown alga *T. conoides*. (A) 24 well plate showing the mosquito larvicidal assay, (B) Mosquito larvae mortality rate and LC_{50} value of the extract.

Table 1 Comparative analysis of LC_{50} values of the marine algal extract against mosquito larvae.

Seaweed	Solvent used	LC_{50} (mg/L)	Reference
Turbinaria conoides	Diethyl ether	104	This study
	Acetone	469	This study
	Benzene	781	This study
	Ethanol	1917	This study
Halimeda macroloba	Ethyl acetate	895	[6]
	Chloroform	1004	[6]
	Methanol	1119	[6]
	Acetone	1110	[6]
	Hexane	1117	[6]
Ulva lactuca	Ethyl acetate	588	[6]
	Chloroform	762	[6]
	Acetone	831	[6]
	Hexane	950	[6]
	Methanol	953	[6]
Caulerpa racemosa	Ethyl acetate	580	[6]
	Chloroform	728	[6]
	Acetone	812	[6]
	Methanol	886	[6]
	Hexane	910	[6]
Turbinaria conoides	Acetone	62	[12]
	Ethanol	75	[12]
	Water	83	[12]
Dictyota linearis	Ethanol 95%	60	[27]
Padina minor	Ethanol 95%	51	[27]
Laurencia dendroidea	2:1 Dichloromethane methanol	100	[27]
Caulerpa racemosa	Ethanol:water (3:1)	0.056	[20]
Ulva fasciata	Benzene	479	[17]
	Acetone	505	[17]
	Methanol	516	[17]
Grateloupia lithophila	Acetone	350	[17]
	Benzene	426	[17]
	Methanol	432	[17]
Lobophora variegata	Methanol	97	[19]
Spatoglossum asperum	Methanol	98	[19]
Stoechospermum marginatum	Methanol	99	[19]
Sargassum wightii	Methanol	87	[19]
Sargassum swartzii	Ethyl acetate	12	[8]
Chondria dasyphylla	Ethyl acetate	11	[8]
Laurencia papillosa	Non polar crude extract	70	[28]
Padina tetrastromatica	1:1 Dichloromethane methanol	100	[29]
Centroceras clavulatum	1:1 Dichloromethane methanol	58	[29]
Microdictyon pseudohapteron	Petroleum ether	50	[30]
Acanthophora muscoides	Petroleum ether	63	[30]
Caulerpa scalpelliformis	Acetone	54	[18]
Enteromorpha intestinalis	Acetone	68	[18]
Ulva lactuca	Acetone	91	[18]
Dictyota dichotoma	Acetone	62	[18]

3.2 Antimicrobial activity

Four different solvent extracts of the *T. conoides* were tested against human pathogen *S. aureu*s by the disc diffusion method. The ZoI was recorded for each extract (Figure 2 and Table 2). Ethanol extract showed the maximum ZoI (17 mm) at the concentration of 3.7 mg (40 µl extract**)** whereas 40 µl ethanol itself shown 10 mm ZoI. Similarly, acetone extract also showed a maximum of 14 mm ZoI at the concentration of 1.2 mg (40 µl extract), whereas 40 µl acetone itself shown 10 mm ZoI. Interestingly, diethyl ether showed 10 mm ZoI with a

concentration of 0.1 mg in 40 µl extract quantity while diethyl ether alone shown 6 mm ZoI. The least antibacterial activity was observed in benzene extract as shown 10 mm with the 2 mg concentration in 40 µl while benzene alone shown 8 mm ZoI. In this, acetone and diethyl ether extract given the same result (4 mm net ZoI) with 3 and 37 times less quantity of extracted biochemical in comparison with ethanol extract (3.7 mg per 40 µl extract), respectively. Therefore, diethyl ether and acetone extract having potential antibacterial compounds.

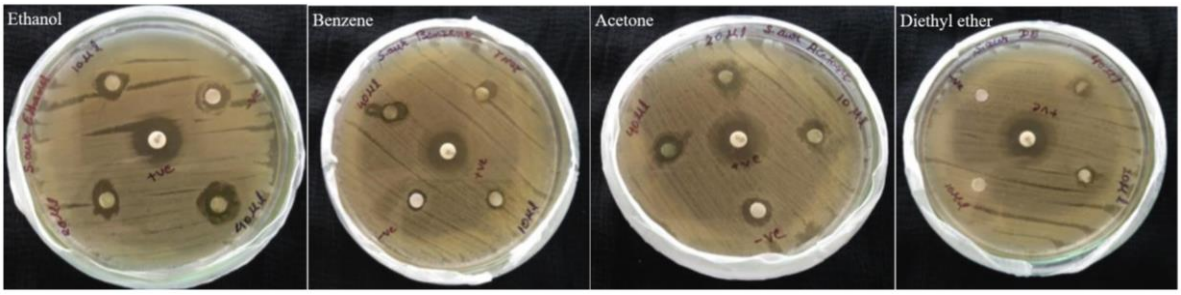

Figure 2 Bactericidal effect against *S. aureus* of the different solvent extract of marine brown alga *T. Conoides.*

Table 2 Zone of inhibition (ø mm) of the different solvent extract of *T. conoides* against *S. aureus.*

Solvent extract	Zone of inhibition (ø mm)				
	10 µl Extract	20 µl Extract	40 µl Extract	+ve control*	-ve control^
Ethanol extract	11	13	17	20	10
Benzene extract	7	9	10	20	8
Acetone extract	10	12	14	20	10
Diethyl ether extract	0	8	10	20	6

*10 mg chloramphenicol disc; ^40µl respective solvent disc.

Intriguingly, no antifungal activity was observed in any of the solvent extracts (Figure 3). The test fungal organism of *A. niger* was grown in all the PDA plates included the 500 µl extract (15 mg of acetone extract, 25 mg of benzene extract, 46 mg of ethanol extract and 1.7 mg of diethyl ether extract) as equal to the control plates (no inclusion of extract). It was indicated that the no antifungal compounds in the marine brown alga *T. conoides* rather it supports the growth of fungi.

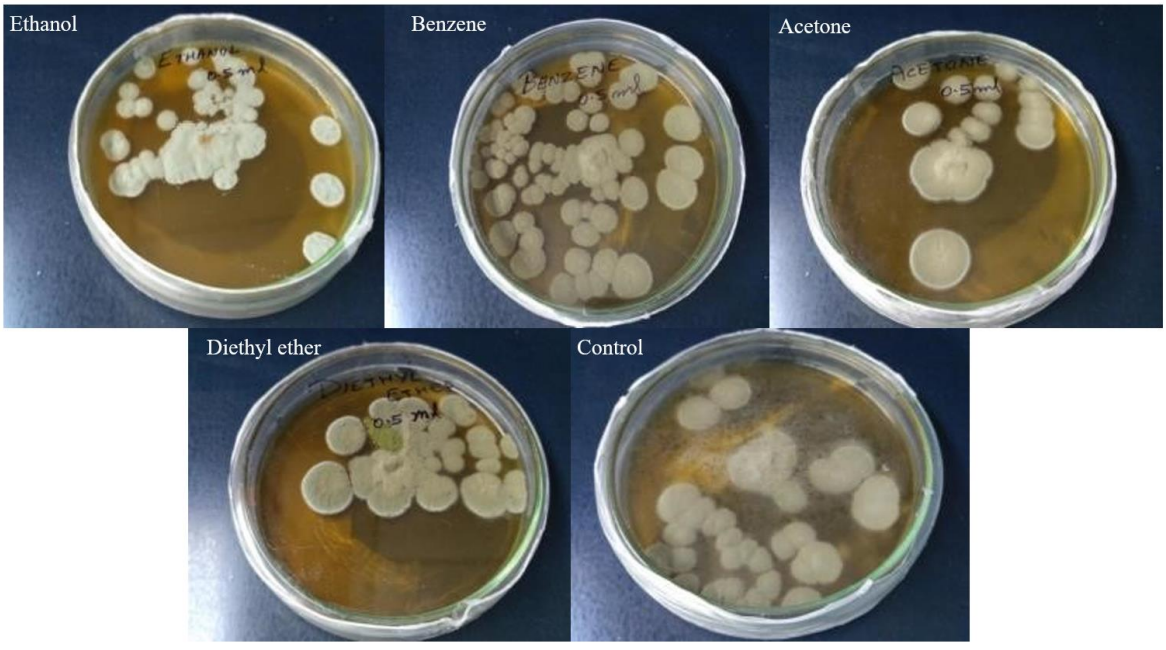

Figure 3 Antifungal action against *A. niger* of different solvent extract of marine brown alga *T. Conoides.*

3.3 GC-MS analysis of the extract

Since acetone and diethyl ether extract of *T. conoides* shown duel action of larvicidal and bactericidal action, hence selected acetone for GC-MS analysis to detect the potential cidal compounds (Figure 4). From the GC-MS data, three major peaks were identified and it was responsible the compounds might liable for the duel activity namely 1,2, Benzenedicarboxylic acid, butyl 8-methylnonyl ester, Pentane 2,2,4-trimethyl, and titanium 1,3,5,7 cyclooctatetraene 2,4 cyclopentadien 1-Yl-. The present study identified that some of these compounds had larvicidal and bactericidal action which will be studied in the future by eluting the particular compound and assayed.

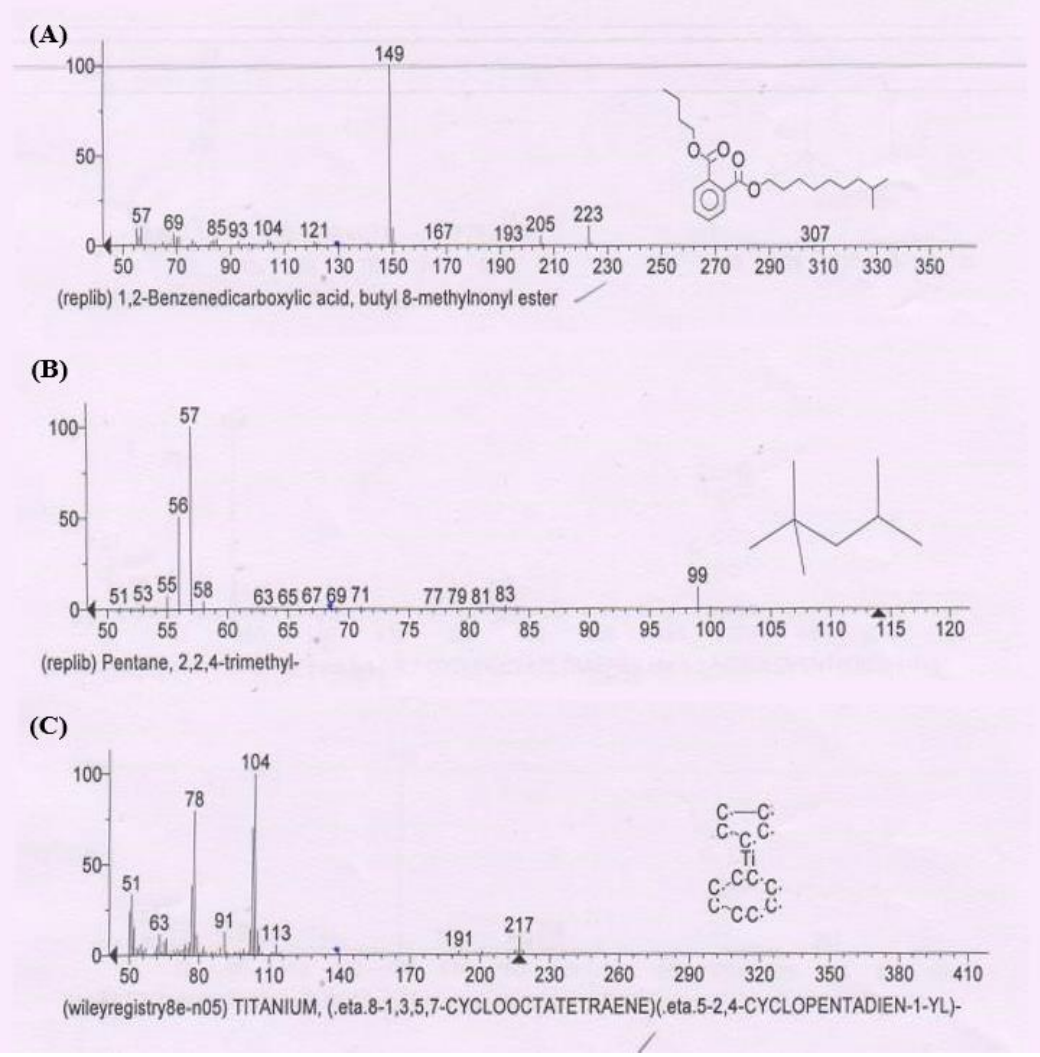

Figure 4 GC-MS chromatogram for acetone extracts of *T. conoides*. Three major chemical compound peaks were identified as 1,2, Benzenedicarboxylic acid, butyl 8-methylnonyl ester (A), Pentane 2,2,4-trimethyl (B), Titanium 1,3,5,7 cyclooctatetraene 2,4 cyclopentadien 1-Yl- (C).

4. Discussion

Though synthetic insecticides and bactericides are effective they create many problems like the development of resistance to insecticide [4] and bactericide [3] and undesirable side effects [13]. Therefore, the usage of indigenous bio-based products could provide a standardized measure for protection to the human population against various diseases caused by mosquito and bacteria. Many approaches have been developed to control the mosquito menace, however, killing its larvae is the paramount approach to prevent mosquito-borne disease. Many studies made use of plant extracts for mosquito larval control [14,15], however very few studies used marine brown algae, recently [6,8,12].

Secondary metabolites with a broad range of activities have been found in marine algae [16]. This study also evidenced mosquito larvicidal and bactericidal compounds in different solvent extracts of marine brown alga of *T. Conoides*. In accordance with this study, Poonguzhali and Nisha [17] observed the same results in acetone extract of seaweed *U. fasciata* and *G. lithophila* with LC_{50} value of 505 mg/L and 350 mg/L for the same mosquito larvae, respectively.

Whereas benzene showed more effective than our study as observed LC_{50} 478 and 425 mg/L, on the contrary, this study noticed at LC_{50} of 781 mg/L respective to benzene extract. Another study found that the aqueous extract of the *T. conoides* shown LC_{50} of 83 mg/L while acetone and ethanol extract had 62 mg/L and 75 mg/L, respectively against *C. quinquefasciatus* larvae, these values were fluctuating with another species of mosquito larvae [12]. The LC_{50} value of acetone extract was very less than our study (469 mg/L), however, both findings supported the acetone extract having the effective compounds. In furtherance to the above findings, recently Adaikalaraj et al. [6] stated that the acetone extract of marine green algae shown mosquito larvicidal activity LC_{50} at 811 mg/L for *C. racemosa* and LC_{50} at 831 mg/L for *U. lactuca*. Moreover, they found ethyl acetate extract was efficient at LC_{50} at 580 mg/L and 588 mg/L for the respective algae, but in our study diethyl ether (104 mg/L) followed by acetone. Conversely, the lowest larval mortality was observed with methanol extract of *H. macroloba* against *Aedes aegypti* with values of LC_{50} at 1119 mg/L [6], but in our study ethanol extract shown at LC_{50} of 1917 mg/L. Interestingly, Thangam and Kathiresan [18] reported that acetone extracts of the marine seaweeds *Caulerpa scapelliformis*, *D. dichotoma*, *Enteromorpha clathrata*, *E. Intestinalis*, and *U. lactuca* were active against fourth instar larvae of *A. aegypti* with LC_{50} values of 53.70, 61.65, 85.11, 67.70 and 91.20 mg/L, respectively. Marine red algae (*Lobophora variegate* (LD_{50} = 95.5 mg/L), *S. asperum* (LD_{50} = 96.1 mg/L) and *S. marginatum* (LD_{50} = 97.3 mg/L) also been reported the mosquito larvicidal activity against third instar larvae of *A. aegypti* [19]. Astoundingly, Ali et al. [20] reported LC_{50} values (0.0556 ± 0.0103) µg/ml, (0.0675±0.1360) µg/ml and (0.0661 ±0.0076) µg/ml, against 4th instar larvae of *A. aegypti*, *C. quinquefasciatus* and *Anopheles stephensi*, respectively using the seaweed extracts of *C. racemosa*. In an, another study on antiplasmodial and antimicrobial activities of South African marine algal extracts, the dichloromethane fraction of *Sargassum heterophyllum* showed the most antiplasmodial effect with LC_{50} value of 2.8 mg/L [21]. It indicates that each solvent is specific for the particular genus of algae and particular species of larvae for the efficient extraction for the bioactive molecules and its action (Table 2).

Increasing resistance of clinically important bacteria to existing antibiotics is a major problem throughout the world [22]. Over the past 20 years, investigators from virtually every corner of the world have documented the increasing proportions of resistance to penicillin and other antibiotics by *S. aureus*. Hence, there has been a great deal of interest in searching for novel natural antibiotics [7,23,24] recently to restrain resistance and this study has shown that organic solvent extract of brown algae *T. conoides* can act as potential antibacterial agents that may be useful in the pharmaceutical industries. All the extract exposed the various extent of inhibition zone against *S. aureus*, however, diethyl ether and acetone extract shown promising antibiotic compounds against *S. Aureus*. Previous studies also reported the potent antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) in marine brown algae *Ecklonia cava* and *Ecklonia Stolonifera* [23,24]. In contradiction to our study, Lee et al. [25] stated that extract of brown alga of *E. cava* has fungicidal activity against *Trichophyton rubrum* associated with dermatophytic nail infections in humans, whereas in our study no antifungal activity was observed against *A. nigar* with any solvent extract of *T. conoides.*

The present study revealed that action of brown seaweed (*T. conoides*) extracts on mosquito larvae (*C. quinquefasciatus*) and *S. aureus* contains a toxic chemical leading to remarkable mortality against its larvae and bacteria. Observations showed that marine brown algae contained a chemical that brought out such mortality to the larvae and bacteria especially in the diethyl ether and the acetone extract. Based on this the acetone extract was analyzed for active dominant compound by GS-MS and observed three major compounds and those compounds will be checked separately for the larvicidal and bactericidal studies in further studies.

5. Conclusion

The present study concludes that the crude solvent (diethyl ether, acetone, ethanol, benzene) extract of *T. conoides* has duel activity of mosquito larvicidal and bactericidal against *C. quinquefasciatus* larvae *and S. aureus* bacteria. Among all extracts, diethyl ether and acetone extract were superior in mosquito larvicidal (LC_{50} 104 mg/L, 469 mg/L) and antibacterial activity (4 mm ZoI at 0.1mg and 1.2mg extract), respectively. Interestingly, no extract showed antifungal activity against *A. niger*. Moreover, there major compounds (1,2, Benzenedicarboxylic acid, butyl 8 methylnonyl ester, Pentane 2,2,4-trimethyl, and titanium 1,3,5,7 cyclooctatetraene 2,4 cyclopentadien 1-Yl-) were identified in acetone extract which might be responsible for the activity. The *T. conoides* extract can be well utilized for preparing larvicidal and bactericide formulation in future with characterization of specific biochemicals in the extract and needed study of non-targeted organisms. In that way, the results of the present

study offer a possible way for further investigations to find out the active molecule needed to elucidate this activity and mechanism against a wide range of action.

6. Acknowledgements

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7. References

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