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Larvicidal activity of microbial metabolites extracted from extremophiles against vector mosquitoes

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Abstract
Mosquitoes transmit disease agents which are responsible for more than 500 million clinical cases estimated by the World Health Organization. The increase in number of resistant varieties of mosquitoes, ineffectiveness of chemical insecticides, necessitates the development of vector control strategies. Thus microbial insecticides can be considered as alternatives to chemical insecticides. In Present study soil and water samples from biodiverse natural habitats such as cold regions and hot water springs were collected and used for screening of bacteria. The extracellular secondary metabolites from the isolates were extracted and tested for its mosquito larvicidal activity against fourth instar larvae of *Culex* and *Aedes* mosquito larvae.

Total 86 soil and water samples were collected from hot water springs, and extremely cold regions of India. 124 bacterial isolates comprising of 24 psychrophiles, 38 mesophiles and 62 thermophiles were obtained. Mosquito larvicidal activity was rendered by 21 isolates and was found to be effective in control of the mosquito larvae. The secondary metabolites were evaluated for its toxicity and the percentage mortality against the larvae was determined. 25% of secondary metabolites showed 100 % mortality against *Culex* at 200 ppm, while 50% of the secondary metabolites showed mortality at 400ppm against *Aedes* larvae. The LC_{50} of the extracted secondary metabolites of the thermophilic isolates was found to be significantly low, about 150ppm. Further efficient thermophilic bacteria were identified biochemically and belonging to *Bacillus* and *Corynebacterium* genera.

Keywords: Extremophiles, Bacillus, Corynebacterium, Culex, Aedes.

1. Introduction
Mosquitoes are insects which are known to harbour plethora of viruses viz. West Nile virus, Saint Louis encephalitis virus, Eastern equine encephalomyelitis virus, Everglades virus, Highlands J virus, Dengue fever, Yellow fever, Ilheus virus, Rift Valley fever and Japanese Encephalitis. There are over 2500 different species of mosquitoes throughout the World. In India three vector-borne diseases namely Malaria, Dengue, and Chikungunya are more prevalent and thus measures to control such mosquito vectors are habitat control, use of insecticides, introduction of sterile male mosquitoes, reduction of the breeding rates and larvicides.

The commercial repellent sprays and mosquito coils use Diethylmetatoluamide (DEET) Methoprene, Briquet, Malathion and Pyrethrum, heavy usage of such chemical agents is proven disadvantageous [1]. Most frequently used chemical agents to inhibit the larval population are Temephos and Fenthion [2].

Development of insecticidal resistance in mosquito populations, inhibition of non-target beneficial insects and contamination of food and drinking water sources leads to damage to the biodiversity and are major drawbacks of overuse of chemical insecticides. At the same time genetic alterations are also contributing to increase in the number of resistant variety of mosquitoes and reducing the effectiveness of insecticides [3].

Microbial insecticides on the other hand; due to their selective toxicity and ready decomposability in the ecosystem, are being considered as alternatives to chemical insecticides. Unlike the inherent dangers associated with the process of production of synthetic insecticides, the process for the manufacture of microbial products is safe, well-contained and less polluted [4]. Microbial metabolites and antimicrobial substances exhibit specific insecticidal activity, bacteria pathogenic to insects such as *Wolbachia* are found to reduce the

susceptibility of *Aedes* mosquito towards the Dengue virus [5]. Similarly *Bacillus thuringiensis*, *Serratia* through their metabolites inhibit *Aedes*, *Anopheles* and *Culex* mosquito larvae [6].

B.subtilis against the larvae of *A.aegypti* produces secondary metabolites which have various mode of action including changes in essential enzymes, AchE, carboxylesterase, alkaline and acid phosphatase of larvae of *A.aegypti*. *B.subtilis* also produces bioactive peptides which include lipopeptides. These are amphiphilic membrane active biosurfactant and peptide antibiotics with potent larvicidal activity [7].

The secondary metabolites produced by Actinobacteria have also shown significant mosquito larvicidal activity against *Culex* species [8]. Tyrosol, 3-methoxy-5-methyl-1,2-benzenediol and 2-hydroxy-4-(4-hydroxychroman-7yl) but-3-enal obtained from extracellular crude extracts of fungi *Stereum* showed strong cidal activity against *A. aegypti* larvae [9].

Yet the microbial world remains the largest unexplored reservoir of biodiversity on the earth. Specifically microbes residing at extreme conditions, these are known to synthesize clinically important metabolites. Hence in the present study regions with extreme hot and cold temperature were selected. The soil and water samples were collected and used for the isolation of bacteria. Bacterial isolates were screened for the potential to inhibit mosquito larvae.

2. Materials and Methods

2.1. Soil and water sample collection

Soil and water samples were collected from hot water springs of Vajreshwari in Maharashtra, Kargil desert and extremely cold regions of Sikkim, Srinagar and forest regions at high altitudes of Kodaikanal and Chikhaldara. The sample collection was carried out for a span of eighteen months (January 2013 to June 2014). Total 86 samples comprising of 74 soil samples and 12 water samples were collected from regions of different ecological niche.

2.2. Isolation of microorganisms

The soil and water samples were enriched in Nutrient broth & incubated at 65 ^{o}C for 48h (Thermophiles), at 10 ^{o}C for a week (Psychrophiles) and at room temperature for 48h (Mesophiles). After enrichment period, one loopful broth was inoculated on to Nutrient agar medium and incubated at respective temperatures. Colonies thus obtained were preserved and were utilized to study their mosquito larvicidal activity.

2.3. Extraction of Secondary Metabolite from the Bacterial Isolates

The secondary metabolites were extracted from bacterial isolates and were inoculated in nutrient broth and incubated at their respective temperatures for 2 weeks [10]. Broth was then filtered, centrifuged at 15000 rpm, for 20 min to obtain cell free supernatant. Equal volume of ethyl acetate was added to the cell free supernatant and kept under shaker conditions for 1 hour. Secondary metabolite was extracted as middle layer after allowing the mixture to settle in separating funnel. The layer of secondary metabolite was collected in a petri dish and dried to evaporation. The dried secondary metabolite was dissolved in dimethyl sulfoxide (DMSO) and used for testing the mosquito larvicidal activity.

2.4. Mosquito larvae rearing

Larvae collected from fields were morphologically identified according to the classification keys provided in photographic manual of mosquito identification [11]. The larvae was reared & used for the study [12].

2.5. Toxicity assay for mosquito larvicidal activity

Isolated bacteria were inoculated in sterile nutrient broth and incubated for 2 weeks. The enriched broth was centrifuged at 15000 rpm for 20 minutes. The cell free supernatant obtained was used for testing its toxicity towards mosquito larvae. For Preliminary testing ten early third instar larvae of mosquito vector *Aedes* were introduced in each of the test solution as well as the control. For each of the dose three replicates were maintained at a time. All the isolates were incubated at room temperature up to 48hrs. Percentage mortality of effective isolates was determined after 24hrs [13] and toxicity assay was carried out for all the bacterial isolates and Temephos 5% (v/v) which was kept as a control.

2.6. LC_{50} determination:

The LC_{50} of the secondary metabolite was determined by using the WHO guidelines 2005 [13]. The concentrations of secondary metabolites were prepared in the range of 100 to 500 ppm. Tests were carried out in 3 batches of 25 third instar larvae in 100ml. Test was conducted in a closed vessel having air space of its 2/3rd volume. This vessel was monitored for next 24 hrs. The minimum concentration inhibiting 50% of the larva population was considered as $LC_{50.}$ The mortalities of treated groups were calculated according to Abbott's formula:

Mortality (%) = [(X – Y)/X] 100,

Where X = % survival in control and Y = % survival in treated mosquitoes.

2.7. Statistical analysis

Twenty five early third instar larvae were introduced in each of the test solution as well as the control. The LC_{50} value and LC_{90} was calculated after 24 hrs by probit analysis [14].

2.8. Identification of microorganisms

Bacterial isolates with mosquito larvicidal activity were identified on the basis of Bergey's manual of determinative bacteriology 9thedition [15]. Their Gram's character, sugar fermentation tests and specific biochemical tests were carried out.

3. Results and Discussion

In past few years resistance to insecticides and chemical agents have been increasing rapidly. Hence there is a persistent demand of developing and searching of new insecticidal agents from natural environments. Extreme natural environments have been consistently generating microbial species which contributes to the control of diseases and their transmission.

In the present study 86 samples were collected from extreme environments. The area selected for sampling were hot water springs of Vajreshwari in Maharashtra, Kargil desert, extremely cold regions of Sikkim, Srinagar and forest regions at high altitudes of Kodaikanal and Chikhaldara. After screening these samples, 124 bacteria which comprises of 24 psychrophiles, 38 mesophiles and 62 thermophiles were obtained. The results are presented in Figure 1

37 bacterial isolates were obtained from Chikhaldara region, while psychrophiles were found in abundance from Southern region of India and Northern Maharashtra. The Kodaikanal

region and Chikhaldara region showed maximum number of thermophiles in the range of 22 and 18 respectively, while psychrophiles were fewer in number around 9 to 6.
From the hot spring regions of Vajreshwari four *Bacillus* spp. were isolated and were found to be effective against *Culex* larvae at LC_{50} of 100 ppm. The forest region of Chikhaldara yielded thermophiles belonging to *Corynebacterium* & *Bacillus* spp.

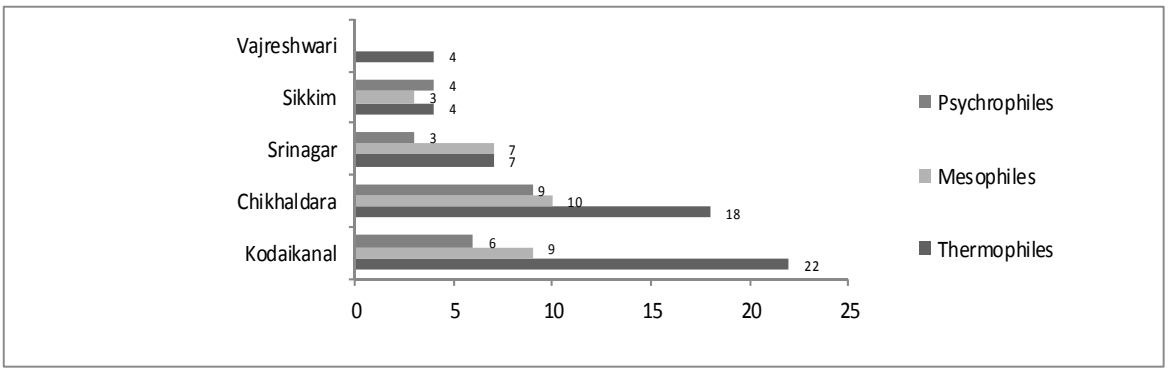

Fig 1: Isolation of extremophiles from different regions and number of isolates obtained

Psychrophilic *Pseudomonas spp* was isolated from Sikkim. Kodaikanal soils showed mixed population of thermophiles, psychrophiles & mesophiles. *Bacillus* was predominant followed by *Pseudomonas* & *Corynebacterium* the results of which are presented in Table 1, which represents the distribution of effective isolates collected from the sampling locations as well as the identification of the isolate upto genus level. These isolates were carefully screened for their potential toxicity towards *Aedes aegypti* and *Culex quinquefasciatus* mosquito larvae. Preliminary study revealed that the 21 bacterial isolates showed mosquito larvicidal activity against *A. aegypti* and *Culex quinquefasciatus* larvae.

Table 1: List of isolates larvicidal against 4th instar *A. aegypti* and *C. quinquefasciatus* mosquito larvae were identified and compared with the area of sampling and their optimum growth character.

Sr. No.	Isolate	Region	Type	Genus of isolate
1	SKP 1	Sikkim	Psychrophile	Pseudomonas
2	SKP 2	Sikkim	Psychrophile	Pseudomonas
3	SKT 1	Sikkim	Thermophile	Bacillus
4	SRP 1	Srinagar	Psychrophile	Bacillus
5	SRT 1	Srinagar	Thermophile	Pseudomonas
6	CKT 1	Chikhaldara	Thermophile	Bacillus
7	CKT 2	Chikhaldara	Thermophile	Corynebacterium
8	CKM 1	Chikhaldara	Mesophile	Bacillus
9	CKP 1	Chikhaldara	Psychrophile	Pseudomonas
10	KP1	Kodaikanal	Psychrophile	Pseudomonas
11	KP 2	Kodaikanal	Psychrophile	Pseudomonas
12	KP3	Kodaikanal	Psychrophile	Corynebacterium
13	KM1	Kodaikanal	Mesophile	Bacillus
14	KM2	Kodaikanal	Mesophile	Bacillus
15	KT1	Kodaikanal	Thermophile	Bacillus
16	VT1	Vajreshwari	Thermophile	Bacillus
17	VT2	Vajreshwari	Thermophile	Bacillus
18	VT3	Vajreshwari	Thermophile	Bacillus
19	VT4	Vajreshwari	Thermophile	Bacillus
20	KARM1	Kargil	Mesophile	Corynebacterium
21	KARM2	Kargil	Mesophile	Bacillus

Secondary metabolites were extracted from the 21 effective isolates given above in table 1.
The bioassay was conducted to estimate the sub lethal mosquito larvicidal concentrations of secondary metabolites. Bioassay was carried out using secondary metabolite in the range of 100 ppm to 500 ppm at the interval of 100 ppm. Total sixteen isolates showed 100 percent mortality at 500 ppm concentration. SKP 2 and SKT 1 showed maximum toxicity towards the *Aedes* mosquito larvae with mortality rate of 80% and 70% respectively, the results are given in Figure 2. While other isolates showed intermediate results with mortality rate in the range of 100 ppm to 400 ppm. In case of *Culex* mosquito larvae, least LC_{50} was found to be 100 ppm, while concentrations up to 400ppm were found to give 100% mortality in 24 hrs. Isolate CKT 2, KT1 and KP2 showed maximum efficacy against *Culex* mosquito larvae in 24hrsas depicted in Figure 2.
The LC_{50} and LC_{90} of the secondary metabolites against 4th instar *A. aegypti* mosquito larvae showed four metabolites having LC_{50} below 100ppm and metabolites of 8 isolates have LC_{90} below 300ppm respectively shown in Table 2. The LC_{50} and LC_{90} of the secondary metabolites against 4th instar *C. quinquefasciatus* mosquito larvae showed three metabolites having LC_{50} below 100 ppm and 7 isolates have LC_{90} below 300ppm. All these isolates were identified up to the genus level using Bergey's manual of determinative bacteriology. It was observed that these 21 isolates were belonging to *Bacillus, Corynebacterium* and *Pseudomonas* genera. Secondary metabolites from *Pseudomonas* spp. were effective against *A. aegypti* larvae, while *Bacillus* was also effective against *C.quinquefasciatus* larvae.
Pseudomonas species have been known to exhibit cytotoxicity against lepidopteran insects as well as mosquito larvae [16, 17, 18]. Migula, an exotoxin released by *P. aeruginosa* acts upon hemolymph proteins of insects. Recently *Pseudomonas* exotoxin based formulation was made to control 4th instar stage larva and pupae of three mosquito vectors of *Aedes*, *Anopheles* and *Culex* genera [19].
Bacillus genus is the most extensively studied genus for the mosquitocidal properties. *Bacillus thuringiensis* and *B. sphaericus* have been used for mosquito control [20, 21, 22]. Other bacilli such as *B. alvei* and *B. brevis* [23], *B. circulans* [24] and *B. subtilis* [25] was also has been reported to produce mosquitocidal toxins. Thus extremophiles are a potential source in control of mosquito larvae.

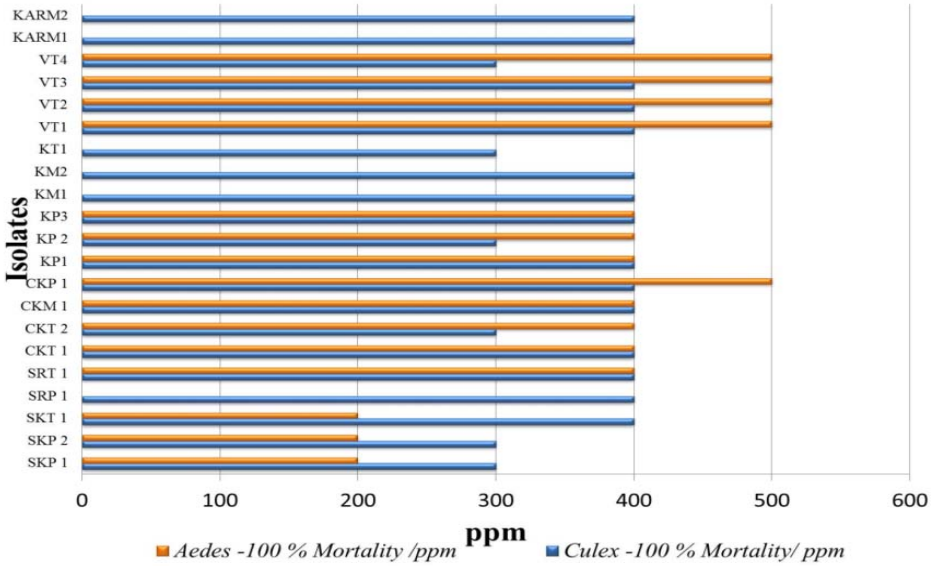

Fig 2: Toxicity and Percentage mortality of secondary metabolites against 4th instar *Aedes* and *Culex* mosquito larvae

Table 2: LC_{50}&LC_{90} of secondary metabolites against 4th instar *C. quinquefasciatus and A.aegypti* larvae in ppm. LC_{50} and LC_{90} values obtained by Probit analysis, Finneys distribution method, α value: 5%, SE: Standard error, p value: <0.05

Isolate Number		A. aegypti					C. quinquefasciatus			Genus
		LC_{50} (ppm)		LC_{90} (ppm)		SE	LC_{50}	LC_{90}	SE	
SKP 1	99	229	5.9	136	299	29.5				*Pseudomonas*
SKP 2	59	203	4.8	105	254	9.8				*Pseudomonas*
SKT 1	62	214	5.3	213	367	48.8				*Bacillus*
SRP 1	367	597	22.4	233	415	55.2				*Bacillus*
SRT 1	267	377	27.5	275	387	69				*Pseudomonas*
CKT 1	210	333	9.2	208	357	49				*Bacillus*
CKT 2	113	293	18.5	84	262	24.1				*Corynebacterium*
CKM 1	210	413	9.9	112	325	36.1				*Bacillus*
CKP 1	119	315	18.5	205	351	36.7				*Pseudomonas*
KP1	126	292	28.1	118	302	19.4				*Pseudomonas*
KP 2	91	244	17.1	124	350	34				*Pseudomonas*
KP3	199	370	40.2	262	364	23				*Corynebacterium*
KM1	116	282	27.9	210	333	9.1				*Bacillus*
KM2	422	613	21.3	156	378	43				*Bacillus*
KT1	290	607	22.1	74	207	13				*Bacillus*
VT1	314	417	9.7	118	302	19.1				*Bacillus*
VT2	310	402	11.1	177	359	67.4				*Bacillus*
VT3	308	398	15.4	175	352	46.2				*Bacillus*
VT4	152	288	7.2	105	254	9.8				*Bacillus*
KARM1	603	865	38.1	641	918	120				*Corynebacterium*
KARM2	491	683	34.2	583	1020	82				*Bacillus*

4. Conclusion

Secondary metabolites from extremophilic microorganisms have immense potential to counter the threat associated to human health from mosquito vectors. The study provided secondary metabolites which are effective in control of *A. Aegypti* and *Culex quinquefasciatus* larvae which are responsible for spread of Dengue and Chikungunya viruses. 100% mortality was obtained by the secondary metabolites isolated from *Bacillus, Pseudomonas* at a concentration of 100ppm. Thus the metabolites can be a potential substitute for the insecticides against which the resistance have already been developed.

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