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Potent targeted larvicidal activities of marine-derived *Bacillus* sp. bacterial extracts on mosquito vectors

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Mosquito vector-borne diseases are one of the leading causes of mortality and morbidity across the globe. Current vector control strategies mainly rely on chemical insecticides, but their incessant usage has resulted in the development of resistance. Insecticidal agents of microbial origin have proven as good alternative tools for vector control of mosquito. In the present study, we examined larvicidal activities of the extracts from culture supernatants of marine bacteria (extracts) against major mosquito vectors from India. Out of 55 tested marine bacterial extracts, 12 extracts caused 90–100% mortality at 250 ppm in *Anopheles stephensi* larvae. Furthermore, NIO 707 and 706 were found to be significantly effective against *Aedes aegypti* larvae and field collected larvae of *An. subpictus*, respectively. Some of the extracts (NIO 701, 707 and 710) demonstrated significant reduction in egg hatching of *An. stephensi*; while all the tested extracts were able to significantly reduce egg hatching in *An. culicifacies*. Additionally, we observed that any of the effective extracts did not show any detrimental activity against malaria parasite (*Plasmodium falciparum*). Phylogenetic analysis revealed that most of the effective extracts belonged to *Bacillus* sp.; however, bacteria from *Enterococcus* genera was a peculiar finding of our study. Altogether, our data underscores the importance of exploration of marine bacteria from Indian peninsula for their larvicidal activities and further undertaking mechanistic approach to develop novel bio-larvicides.

Keywords Marine bacteria, Culture supernatants, Malaria, Insecticide resistance, Larvicidal, *Anopheles*, *Aedes*

Mosquitoes serve as potent vectors for a multitude of diseases, notably malaria, dengue, chikungunya, filariasis, leishmaniasis, and japanese encephalitis. The burden of mosquito borne diseases is a global health problem and affects more than 40% of the world's population[1]. Around half of the globe's population is at risk of dengue infection, with WHO estimating 100–400 million dengue cases each year[2]. According to world malaria report 2023, there were an estimated 249 million malaria cases, globally and in the past one decade, 88 countries have reported insecticide resistance amongst the vector populations[3]. Successful vector control strategies employ long lasting insecticide-treated bed-nets (LLINs) and indoor-residual spray (IRS), where pyrethroids are most widely used for treating LLINs and for IRS[4,5]. However, continuous application of pyrethroids has resulted in emergence of resistance among mosquito vector populations[6]. Thus, necessitating the development of novel insecticidal molecules or strategies to combat the looming challenge of resistance. Moreover, these approaches encounter additional obstacles, such as elevated costs and concerns about toxicity, environmental hazard, etc.

Unlike synthetic insecticides, majority of bio-insecticides are derived from microbial sources and have proven to be an effective alternative strategy for vector control[4,7,8]. Bio-insecticides can include plant-incorporated protectants, microbial pesticides, phytochemicals, and pheromones. These alternatives exhibit lower toxicity, greater specificity, biodegradability, require lesser quantities, and are less prone to develop resistance by insects[9,10]. *Bacillus thuringiensis israelensis* (Bti) and *Lysinibacillus sphaer* extracts were found to effectively kill the larvae of multiple mosquito species[11]. Similarly, larvivorous fishes in aquatic water bodies reduced the

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larval population, thereby abolishing the development of adult mosquitoes[12,13]. Furthermore, certain bacterial extracts and isolated compounds have demonstrated larvicidal activity. Notably, spinosyn A and spinosyn D, derived from the bacterium *Saccharopolyspora spinosa* from *Actinomycetes*, were found effective against various mosquito species including *An. gambiae* and *An. funestus*[14], *An. dirus*, *An. minimus*[15]. Field-tested formulations of spinosad have been found to be effective against *Culex quinquefasciatus*, a vector of filariasis in India[16]. Essential oils have also shown promise as a valuable source of bio-larvicides against *Ae. aegypti*[17–19]. Crude leaf extracts of *Momordica foetida*, *Calpurnia aurea* and *Zehneria scabra* displayed low IC_{50} values of 35 ppm against *An. stephensi*[20]. Additionally, *Saussurea costus* extract exhibited significant activity against three major mosquito vectors (*Aedes*, *Anopheles* and *Culex*), with an IC_{50} value ~ 8 ppm against *An. stephensi*[21]. Numerous ethnobotanical plants have demonstrated larvicidal and adulticidal properties against *An. arabiensis* with notable examples including *Ocimum lamiifolium*, *Ocimum americanum*, *Azadirachta indica*, *Moringa olifeira* leaf and seed species[22].

Previous studies have showed bacterium *Bacillus* and its isolates as efficient bio-larvicides against mosquito vectors. For example, *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* have been popularly used as an effective larvicide for a long time, with minimum non-target effects on other organisms[23]. A recent study showed the supernatants of *Bacillus safensis* Bac I67 and *Bacillus paranthracis* C21 efficiently killed the *Aedes aegypti* larvae from a cohort of 254 different bacterial extracts[24]. Similarly, Bis-(2-ethylhexyl) phthalate from *Lactiplantibacillus plantarum* was reported to have anti-larval activity against *Culex* sp[25]. Metabolites isolated from *Nocardia* and *Streptomyces* were tested against the fourth stage larvae of *Anopheles* stages and were found to be effective with LC_{50} ranging from 300 to 600 ppm[25]. All these studies indicated that bacteria-derived larvicides are effective and could be exploited for mosquito larval control. In context of the larvicidal activities from marine bacteria have not gained much attention. Crude extracts of the marine bacteria *Streptomyces* sp. exhibited antilarval activity against three different disease vectors (*An. stephensi*, *Culex tritaeniorhynchus* and *Rhipicephalus microplus*). One of the purified marine actinobacterial compound DMBPO had displayed LC_{50} values of 88.97 ppm against the *An. stephensi*[26]. In the present exploratory study, we investigated the larvicidal activities of the extracts from culture supernatants of various marine bacteria (denoted as 'extracts' further on in the study) collected from various locations across Indian peninsula.

Results

Larvicidal activities of extracts from marine bacteria on mosquito vectors

We examined the larvicidal activities of the 55 marine bacterial extracts against the larvae of *An. stephensi* (Fig. 1A). Amongst 55 extracts, 12 extracts (NIO 97, 116, 124, 132, 258, 276, 311, 701, 706, 707, 710, 718) were found effective against *An. stephensi* larvae at concentrations (250 and 125 ppm) (Fig. 1A). Further, the effective extracts were assessed for their dose-dependent larvicidal activities against *An. stephensi* larvae (250–7.5 ppm) (Fig. 1B). Except NIO 132 and 707; all extracts exhibited significant larval mortality (> 50%) at 62 ppm compared to control. Using PROBIT analysis, the LC_{50} values and fiducial limits of 12 extracts were calculated and summarized in Table 1. Unfortunately, estimation of the larvicidal activities required large amounts of extracts; during the course of experiments, some of the extracts exhausted and could not be continued for further investigation. Therefore, extracts NIO 97, 124, 276, 701, 707 and 710 were tested for their larvicidal activities against *Ae. aegypti* larvae at two concentrations (500 and 250 ppm) (Fig. 2A). Only NIO 707 extract demonstrated > 70% mortality for *Ae. aegypti* larvae at 500 ppm (Fig. 2A). To check the effectiveness of the extracts in a semi-field setting, *An. subpictus* larvae were collected from the NIMR field unit at Mewat, Haryana, India. Available effective extracts (NIO 97, 124, 276, 701 and 706) were used at a concentration of 125 ppm against the field collected larvae of *An. subpictus*. Among the tested extracts, only NIO 706, showed > 80% mortality at 125 ppm concentration in 24 h. (Fig. 2B).

Reduced egg hatching in *Anopheles*

The eggs of *An. stephensi* and *An. culicifacies* were incubated with the extracts to check their action on the hatchability of the eggs. Reduced transformation of the eggs to the larvae was observed in both the *Anopheles* vectors (Fig. 3A,B). In case of *An. stephensi*, extracts NIO 701, 706 and 707 found to reduce the hatchability of the eggs at 500 ppm, compared to control. However, for *An. culicifacies*, all extracts at 500 ppm (NIO 97, 124, 276, 701, 706, 707 and 710) were effective in reducing the number of eggs transformed to larvae, compared to control.

Phylogenetic analysis of the larvicidal extracts

Phylogenetic analysis of the extracts subjected to antilarval activities against different mosquito vectors and reduction in egg hatching was performed. 16S rRNA identification for NIO 97, 124, 276, 701, 706, 707 and 710 was performed and a phylogenetic tree was constructed (Fig. 4). The locations of the collection sites of marine bacteria included in the phylogenetic analysis; their closest strains and sequence identities are summarized in Table 2. Phylogenetic analysis revealed that most of the marine bacteria were isolates of *Bacillus* sp. (NIO 97, 124, 701, 706 and 707). However, we observed that the larvicidal activity of NIO 276 (*Enterococcus casseliflavus*) extract was a peculiar finding of this study.

Assessment of the antiparasitic activity of the extracts

To examine the non-specific activity of selected marine extracts, we performed *P. falciparum* parasite growth inhibition assay. Notably, except NIO 97, none of the tested extracts showed any significant detrimental effect on parasite growth at concentration of 50 µg/ml (Fig. 5A). The treated parasites were observed to have similar growth pattern as untreated and methanol exposed control parasites. The parasites treated with the extracts displayed similar parasitemia and morphology as compared to control (Fig. 5B). We used chloroquine (CQ) as

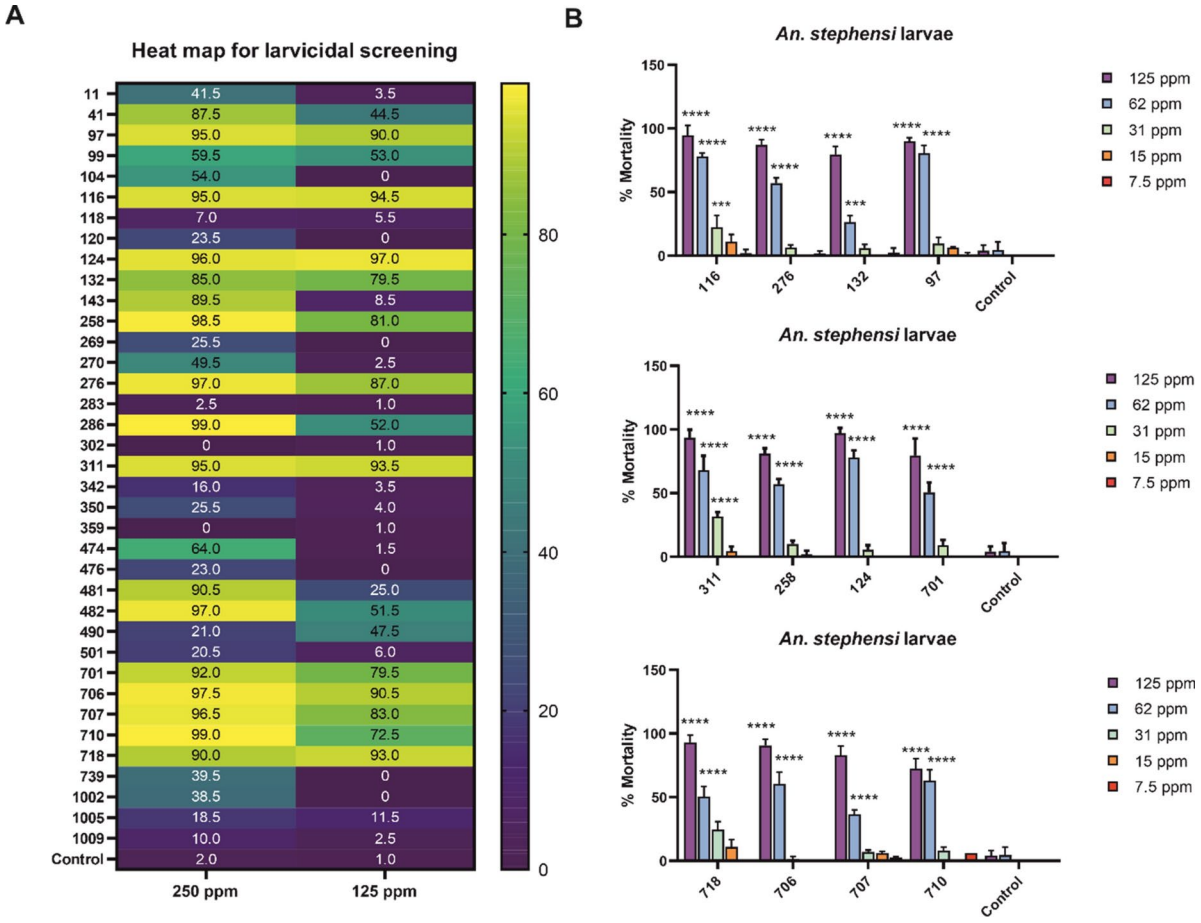

Fig. 1. Larvicidal activities of extracts of marine bacteria against *An. stephensi* larvae. (**A**) L3-stage (n = 10) *An. stephensi* larvae were treated with different marine extracts (250 ppm and 125 ppm). (**B**) Effective marine extracts (> 90% mortality) were tested at lower concentrations in dose dependent assays. Two-way Annova statistical test; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0003$, ****$p < 0.0001$. Data is representative of two independent experiments.

Name of extract	LC_{50} (Fiducial limits) [values in ppm]
NIO 116	44.78 (31.35–63.97)
NIO 276	50.7 (35.5–72.32)
NIO 132	83.85 (56.14–125.24)
NIO 97	52.28 (36.94–73.98)
NIO 311	56.05 (38.21–82.21)
NIO 258	63.81 (46.14–88.23)
NIO 124	59.8 (44.07–81.24)
NIO 701	72.6 (51.75–102.02)
NIO 718	55.41 (37.74–81.35)
NIO 706	71.21 (53.60–94.5)
NIO 707	59.76 (41.68–87.70)
NIO 710	64.8 (42.36–99.20)

Table 1. LC_{50} and fiducial limits of the extracts from culture supernatants of marine bacteria using PROBIT analysis.

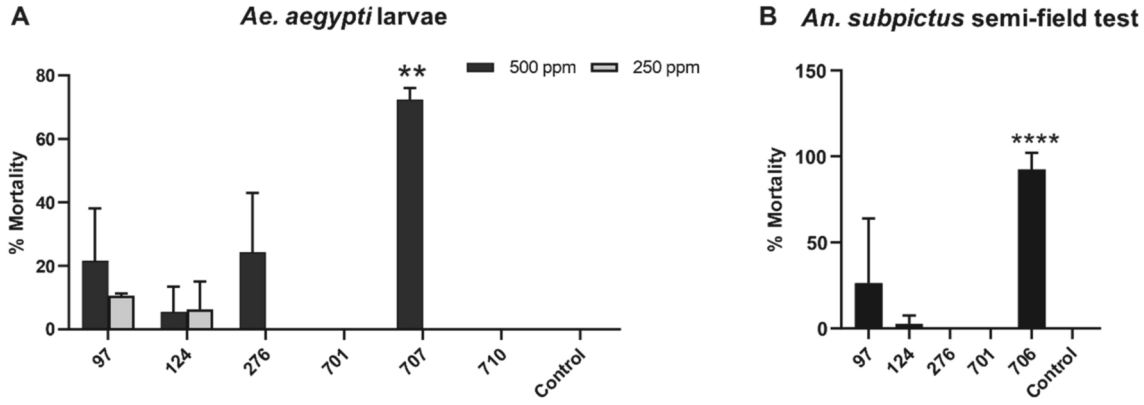

Fig. 2. Larvicidal activities of few bacterial extracts on *Ae. aegypti* and *An. subpictus* larvae. (**A**) NIO 707 (*Bacillus* sp.) exhibited anti-larval action against the dengue vector, *Aedes aegypti*. (**B**) In the semi-field study, NIO 706 (*Bacillus amyloliquefaciens* strain B9) displayed larvicidal activity in *An. subpictus* collected from Mewat, Haryana. One-way Annova statistical test; *p < 0.05, **p < 0.001, ***p < 0.0003, ****p < 0.0001. Data is representative of two independent experiments.

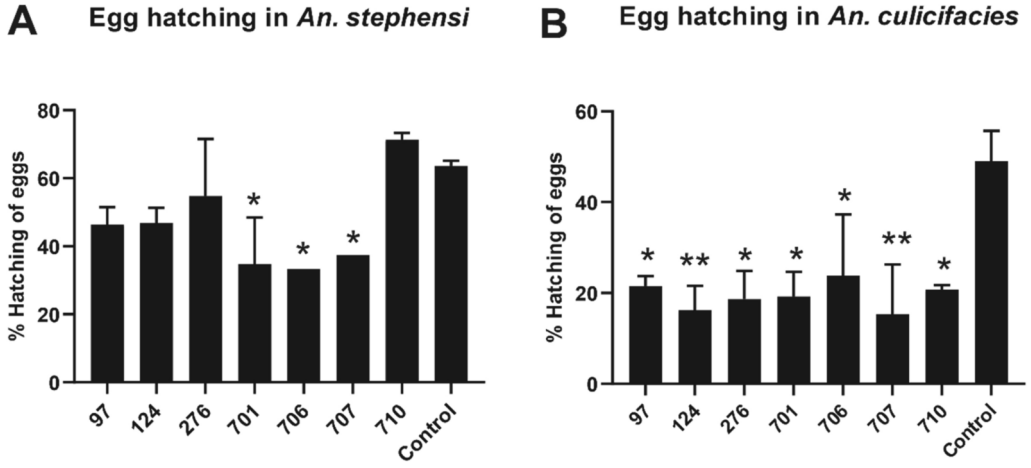

Fig. 3. Reduction in egg hatchability. (**A**) Bar graph showing reduction in egg hatchability post exposure to marine bacterial extracts in *An. stephensi*. (**B**) Bar graph showing reduction in egg hatchability in *An. culicifacies*. Data is representative of two independent experiments; One-way Annova statistical test; *p < 0.05, **p < 0.001, ***p < 0.0003, ****p < 0.0001.

positive control in the growth inhibition assay with very few or no parasites after CQ treatment after 24 h. of exposure.

Discussion

The emergence of resistance against the conventional chemical insecticides by mosquitoes and the associated public health risks have spurred the search for safer, eco-friendly, and more economical options. For mosquito vector control, microbes-derived larvicides have proven as promising alternatives to conventional insecticides. In this study, we explored *An. stephensi* larvicidal activities of extracts from the culture supernatants of marine bacteria (n = 55) collected from different Indian coastal regions. 12 extracts were found to possess larvicidal activities. Unfortunately, due to requirement of large quantities of extracts in larvicidal screening assays, many extracts exhausted and could not be continued further for investigation. Subsequently, the available extracts were further subjected to larvicidal assays in other mosquito vectors such as *Ae. aegypti*, *An. subpictus* in semi-field setting; and impact on egg hatching in *An. stephensi* and *An. culicifacies*, respectively. Interestingly, there was no significant anti-parasitic activities observed in the extracts against *P. falciparum*.

It was interesting to observe that extracts (NIO 97, 124, 276, 701 and 710) were effective against *An. stephensi* larvae, but except NIO 707 none of the effective extracts demonstrated larvicidal activity against *Ae. aegypti*. Moreover, when the effective extracts were tested in semi-field setting on *An. subpictus* larvae, only NIO 706 was found effective. The difference in larvicidal activities on lab reared larvae and field collected larvae might be attributed to the physiological adaptation/resistance, because later larvae dwell in pond/natural water bodies along with other bacteria and organisms. The LC_{50} values indicating the larvicidal activity against *An. stephensi*

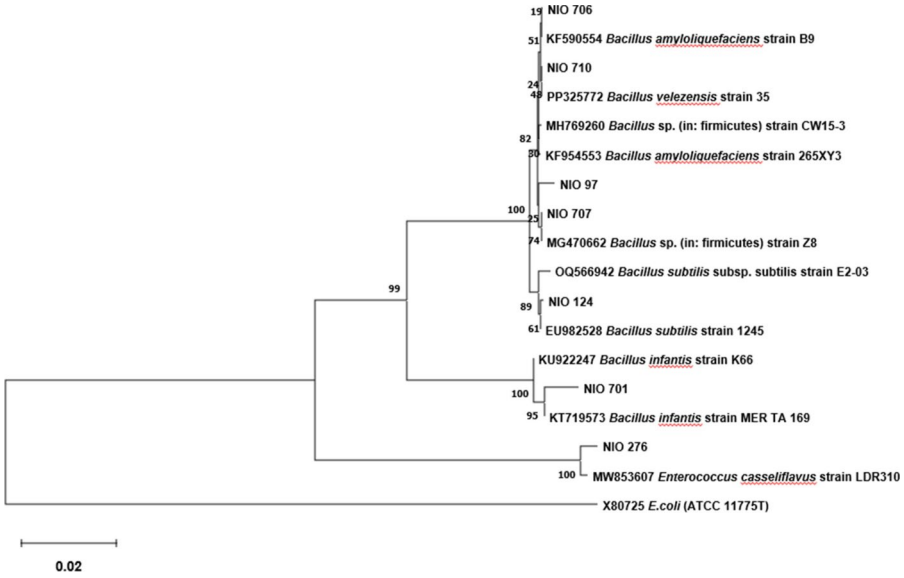

Fig. 4. Phylogenetic tree of the marine bacteria with larvicidal activities. The tree was generated using the Neighbor Joining method with 1000 bootstrap replications. *Escherichia coli* (ATCC 11775 T) was taken as the outgroup. The number on the clads represents the phylogenetic difference.

Sr. no	Isolate ID	Source	Location	Closest type strain and NCBI accession number	Sequence identity (%)
1	NIO 97	Coral (*Lobophytum crassum*)	Lakshadweep (Kavaratti)	*Bacillus amyloliquefaciens* strain 265XY3	98.26
2	NIO 124	Sponge (*Cinachyrella* sp.)	Ratnagiri	*Bacillus subtilis* strain 1245	99.48
3	NIO 276	Sponge	Okha	*Enterococcus casseliflavus* strain LDR310	99.67
4	NIO 701	Seaweed	Okha	*Bacillus infantis* strain MER_TA_169	98.80
5	NIO 706	Seaweed	Okha	*Bacillus amyloliquefaciens* strain B9	100
6	NIO 707	Seaweed	Okha	*Bacillus* sp. (in: Bacteria) strain Z8	100
7	NIO 710	Seaweed	Okha	*Bacillus velezensis* strain 35	100

Table 2. Phylogenetic analysis of bacterial extracts using 16S rRNA sequencing.

are in the range of 44–83 ppm, which is considered potent for whole extracts. Further, the active chemical constituents upon characterization might be more effective compared to whole extracts. These results point towards targeted action of the chemical constituents present in different extracts. Such differential larvicidal activities of extracts against different mosquito species warrant further elucidation in future studies. The effective extracts were tested for their potential to reduce eggs hatching in *An. stephensi* and *An. culicifacies*. Interestingly, for *An. stephensi*, only extracts NIO 701, 706 and 707 impacted egg hatching, while in *An. culicifacies*, all tested extracts significantly reduced egg hatching; pointing towards selective action of the chemical constituents in the extracts, warranting further elucidation.

Phylogenetic analysis of the extracts tested for larvicidal as well as egg hatching impact, demonstrated that majority of the effective extracts belonged to *Bacillus* sp. Although, the elucidation of the chemical constituents imparting larvicidal activities needs mechanistic characterization, which is beyond the scope of current study. Interestingly, one extract NIO 276 having larvicidal activity against *An. stephensi* larvae, belonged to *Enterococcus* genera.

Importantly, for any larvicidal product to be useful for ground implementation, a detailed characterization with respect to its ecological impact and toxicological profiles have to be assessed thoroughly, along with its chemical characterization. Novel formulations with other currently used insecticides or other potent bio-larvicides should be empirically determined and validated in field settings. The present study provided concrete evidence for the larvicidal activities of bacterial extracts collected from marine bodies of India and warrants further exploration for more bacterial species.

Methodology

Preparation of the extracts of culture supernatants from marine bacteria

Bacteria were collected from different locations of marine bodies across Indian peninsula, namely-Gujarat, Maharashtra, Karnataka, Goa, Tamil Nadu and Lakshadweep (Kavaratti) (Table 2). The bacterial isolates were collected from marine invertebrates and sediments etc. The isolates were sub-cultured on Zobell Marine Agar till pure colonies were obtained. A loopful of the grown bacterial culture was inoculated in Zobell Marine Broth and

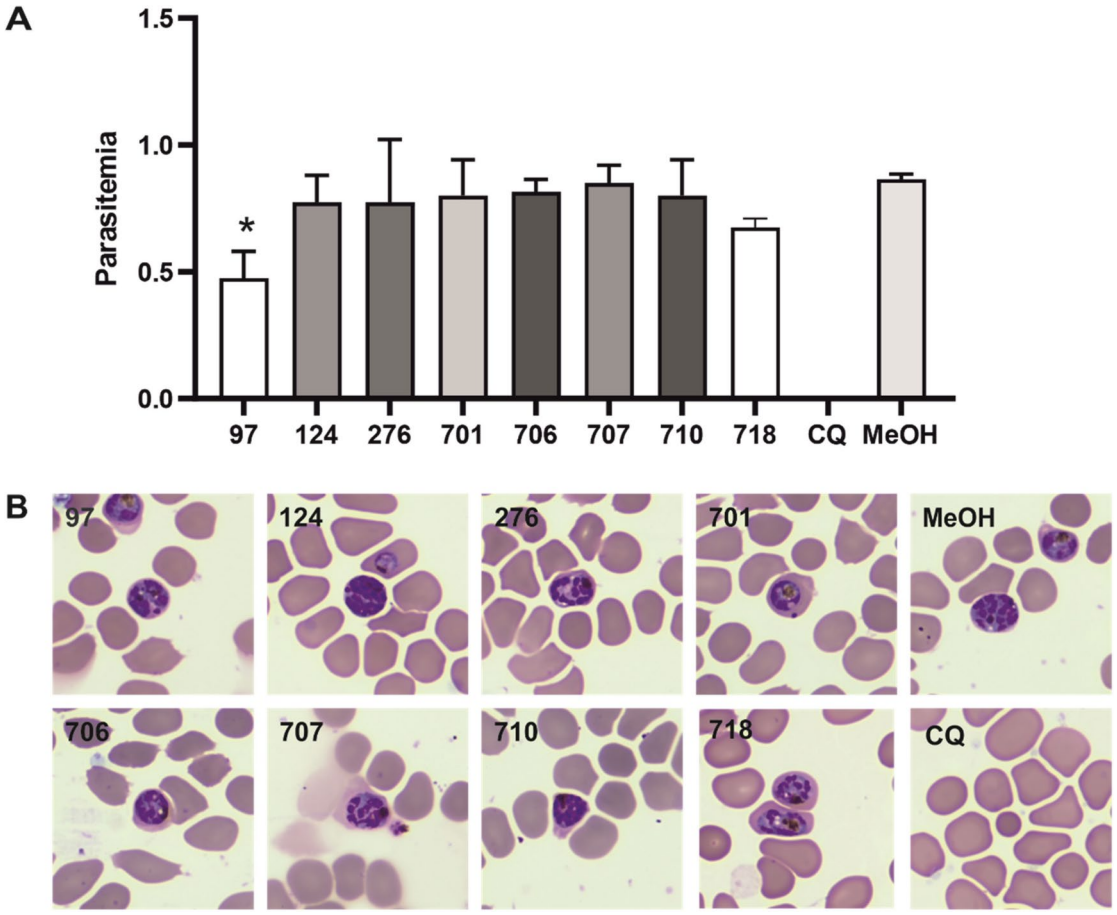

Fig. 5. Anti-plasmodial assay of few effective bacterial extracts against *P. falciparum*. (**A**) Bar graphs showing the reduction in parasitemia of *P. falciparum* post treatment with 50 µg/ml of extracts after 24 h. (**B**) Thin blood smears of the *P. falciparum* culture after exposure to various bacterial extracts showing no significant impact. One-way Annova statistical test; *p < 0.05, **p < 0.001, ***p < 0.0003, ****p < 0.0001. *MeOH* Methanol, *CQ* Chloroquine.

incubated at 35 °C and 100 rpm for 96 h. The culture was then centrifuged at 10,000 rpm for 15 min to obtain the supernatant and pellet. The chemical constituents from the supernatant were extracted thrice with Ethyl acetate (A.R Grade). The solvent fractions were pooled together and concentrated on a rotary vacuum evaporator to obtain the final crude extract.

Phylogenetic analysis

The genomic DNA of bacterial isolates for 16S rRNA gene sequencing was extracted using Himedia HiPurA® Bacterial Genomic DNA Purification Kit. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used. Polymerase Chain Reaction (PCR) was carried out in Takara Thermal cycler Dice TP600 Gradient PCR. The reaction mixture consisted of 12.5 µl of Promega GoTaq Green Master Mix (2×), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of 100 ng DNA template, 9.5 µl of Nuclease free water, making a total reaction volume of 25 µl. The PCR conditions were as follows: Denaturation at 95 °C for 3 min, followed by 95 °C for 1 min; annealing at 52 °C for 30 s; extension at 72 °C for 2 min (30 cycles) and final extension at 72 °C for 10 min. The amplified products were cleaned using Promega Wizard® SV Gel and PCR Clean-Up System. The PCR purified products/samples were sent to Barcode Biosciences (Bangalore, India) for sequencing. The forward and reverse sequence of 16 s rRNA were trimmed manually by using Bioedit Sequence Alignment Editor Software (Freeware, CA, USA)[27]. Basic Local Alignment Search Tool [BLAST] from National Centre for Biotechnology Information (NCBI) was used to find the identities of the isolates. A phylogenetic tree was constructed by MEGA 11 software[28] by neighbour joining method using 1000 bootstrap replicates.

Mosquito rearing

The mosquito strains used in this study were reared and maintained at the insectariums of the ICMR-National Institute of Malaria Research, New Delhi, India. The mosquito strains for the semi-field study were collected from Mewat, Haryana, India. The larvae were maintained at a temperature of 27 ± 2 °C and with a relative humidity of 78 ± 2%. The larvae were fed with a mixture of dog food (Pet Lovers Crunch Veg) and fish food

(Tetra Bits Complete fish food). The larvae were fed and reared under the above-mentioned conditions until they turned stage three instars (L3) and were taken for screening as per WHO guidelines[29] and previously reported studies[30,31].

Dose-dependent larval bioassay

The larvicidal activity of the extracts were assessed by exposing L3 larvae of the *Anopheles stephensi* to the marine bacterial culture supernatant extracts. The bioassays were conducted at 27 ± 3 °C, 80 ± 3% relative humidity (RH), with six replicates per extract, including a positive control (temephos) and negative control (water). The % mortality were counted at the concentrations of 250 ppm and 125 ppm of extracts[32,33]. Selected hits from the preliminary screening were taken for further dose-dependent bioassays with lower concentrations starting from 125 to 7.5 ppm. LC_{50} and fiducial limits of hits were calculated according to the PROBIT statistical analysis.

Egg hatchability

Selected extracts (NIO 97, 124, 276, 701, 706, 707, 710) at 500 ppm were added to the water in a bowl with 100 eggs of *An. stephensi*. The number of eggs were counted at the beginning and at the end of the experiment, number of live larvae in treated and control groups were counted. The percent egg hatchability was calculated as number of live larvae divided by total number of eggs exposed[33,34].

In vitro *P. falciparum* growth inhibition assay

P. falciparum strain 3D7 was cultured in fresh A + human erythrocytes at 5% hematocrit with 10.4 g/l RPMI 1640 (Gibco), pH 7.2, 0.5% AlbuMAX II (Gibco), 5% sodium bicarbonate (Sigma) supplemented with 50 mg/ml gentamycin and 50 mg/l hypoxanthine and incubated at 5% CO_2 and 37 °C. For general maintenance of culture, parasites were grown in a non-synchronous manner with parasitemia between 0.2 to 10% and monitored regularly. *P. falciparum* 3D7 cultures were synchronized using 5% sorbitol and parasitemia was assessed. Parasites with synchronized ring stages at 0.5% parasitemia were then added to 96-well plates with 5% hematocrit. Different concentrations of the extracts (50 µg/mL and 1 µg/mL) were incubated with the culture, as mentioned above for 24 h. Parasite growth was assessed by reading thin blood smears made by fixing in 100% methanol and stained for 20–30 min in 10% Giemsa stain solution. Chloroquine and Methanol were used as negative and positive controls, respectively. Experiment was performed in duplicates with two different biological replicates[35–37].

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

Conceived the study-K.C.P., K.V., N.T. Data generation, interpretation-C.P., H.D., V.V., M.P.K., K.V. Manuscript writing and review-C.P., K.V., O.P.S., N.T., K.C.P., R.D.

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Competing interests

The authors declare no competing interests.

Additional information

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