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Potentiation of *Bacillus thuringiensis* by using some natural products: Novel preparations against dengue vector *Aedes aegypti* larvae

Chandrakant P Narkhede[1], Sunil H Koli[1], Rahul K Suryawanshi[1], Chandrashekhar D Patil[1], Hemant P Borase [1], Satish V Patil[1,2*]

[1]School of Life Sciences, [2] North Maharashtra Microbial Culture Collection Centre (NMCC), North Maharashtra University, Post Box 80, Jalgaon 425001, Maharashtra, India

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Dengue fever is the fastest emerging arboviral infection causing millions of deaths all over the world. The eradication of vector *Aedes aegypti*, is an effective method of dengue control. Although various vector control agents like chemical pesticides are available, *Bacillus thuringiensis* (Bt) is of major choice as a biocontrol agent due to its ecofriendly nature. In the present investigation, curcumin, plumbagin, camphor, rutin, quercetin, karanjin, and pongamal were used as Bt SV2 potentiating agents. It was observed that curcumin and rutin had very high LC_{50} values for fourth instar larvae of *Ae. aegypti* that indicates lower activity. Karanjin caused significantly high mortality at comparatively low dose (LC_{50} - 44.59 ppm). At the same time pongamal, plumbagin, and camphor caused significant mortality at low doses of LC_{50} 61.18, 59.23, and 71.59 ppm, respectively.

Keywords: *Aedes aegypti*, Bti, Camphor, Combination, Natural product, Plumbagin, Potentiation.

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Introduction

Dengue fever is the fastest emerging arboviral infection spread by major insect vector *Aedes aegypti*, which leads to major public health consequences in over 100 tropical and sub-tropical countries in South-East Asia, Western Pacific as well as South and Central America[1]. As per WHO report, worldwide 2.5 billion people live under the threat of dengue fever including dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). It was estimated that out of 50 million cases of dengue fever, half a million people suffering from hemorrhagic fever require hospitalization each year and about 2.5 % of dengue infected population die[1].

Due to lack of specific drugs or vaccines for prevention and treatment of dengue infection, eradication of insect vector namely *Ae. aegypti*, may be considered as the prime method to control dengue infection. Besides the chemical pesticides, current use of biocontrol agents *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Bs), are reported to be effective larvicide. They do not affect the non-target organisms and have eco friendly nature. Bt has been found to show very low mammalian toxicity and therefore, been recommended for household use to control mosquitoes[2,3]. Dengue vector control programs majorly advocates the use of larvicide rather than the space spraying because of its drawbacks like need of specific operations, photo inactivation possibility, and its economical feasibility. Spraying of larvicides also has limited success due to public unacceptability and variable degree of compliance by the communities[1]. Besides these challenges, insecticide resistance to Bt is one of the major threats to the effectiveness of vector control programs.

To achieve sustainability in dengue vector control programme, it is essential to focus on the development of new multi target formulations for the reduction of larval population. In the present investigation, potentiation of Bt SV2 with some natural products is proposed. These natural products include i) Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside), ii) Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one), iii) Curcumin, iv) Plumbagin, v) Camphor, vi) Pongamol, and vii) Karanjin (Fig. 1). Rutin and quercetin categorized as flavonoids are reported for their pharmacological properties like antioxidative, antimicrobial, antifungal, and anti allergic[4]. Rutin has potent

*Correspondent author
Email: satish.patil7@gmail.com

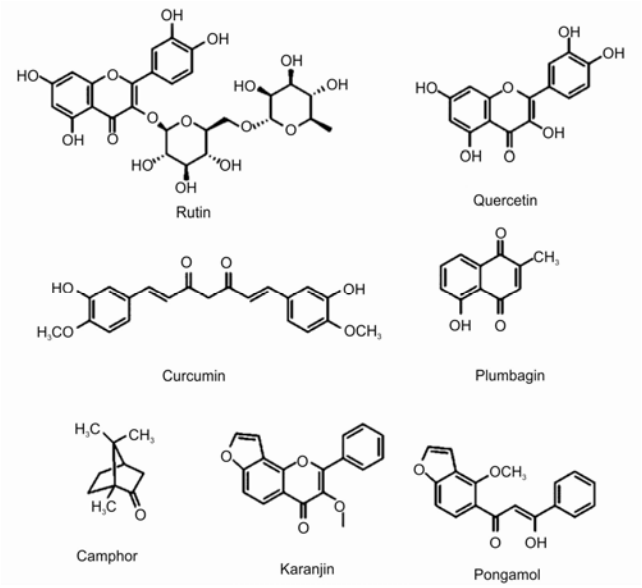

Fig. 1—Structures of natural products used for potentiation of Bt SV2

anthelmintic, larvicidal, and cytotoxic potential[5]. Curcumin is one of the curcuminoids that is part of turmeric[6]. Curcuminoids are well documented for their antimicrobial activity and free radical-scavenging potential[7,8]. *Plumbago zeylanica*, belonging to family Plumbaginaceae is a tropical shrub with activity reported against intestinal parasites, scabies, and treatment of rheumatism and swelling[9]. Camphor has traditionally been used as antiseptic, analgesic, and antipruritic[10,11]. Various plant parts of *Pongamia pinnata* have been reported in treatment of tumors, piles, skin diseases, wounds, and ulcers. The bioactive molecules of the plant extract contain abundant prenylated flavonoids such as furanoflavones, furanoflavonols, chromenoflavones, furanochalcones, and pyranochalcones. Karanjin, a major component of *Karanja* (*P. pinnata*) seed oil has been reported as antifeedant and insect repellant[12].

Materials and Methods

Fine chemicals and reagents

Curcumin, plumbagin, camphor, rutin, quercetin, karanjin, and pongamol were obtained from Hi Media, Mumbai, India / Sigma Aldrich, Mumbai.

Bt compatibility with natural product

The previously isolated and tested bacterial strain Bt SV2 (*B. thuringiensis* species: JN315886 strain) was evaluated for its compatibility with natural product at 10 mg % concentration by incorporating individually in sterilized nutrient agar. These plates were streaked with 24 h old Bt SV2 culture and incubated for 24 h at 30 °C and standard bacterial strain *B. thuringiensis* subsp. *israelensis* H 14 (Bti) was isolated from the commercial *B. thuringiensis* spore-crystal formulation, Vectobac and used as reference strain[13].

Mosquito cultures

Fourth instar larvae of *Ae. aegypti* were maintained as described by Patil *et al.*, that is the larvae were kept in glass trays, containing distilled water at 28±2 °C and 75–85 % relative humidity under 14:10 light and dark cycles[13]. They were fed with a diet of finely ground yeast powder (Hi media, India) and dog biscuits in a ratio of 3:1.

Larvicidal assay

The larvicidal assays were performed by exposing them to lyophilized Bt SV2 in combination (1:1) with curcumin, plumbagin, camphor, karanjin, and pongamol at differential concentrations ranging between 10 to 100 ppm and for rutin, quercetin 100 to 1000 ppm. Batches of 20 fourth instar larvae were introduced in 100 mL test medium containing a respective test concentrations of combinations or tap water alone (control). All containers were maintained at room temperature with naturally prevailing photoperiod in the laboratory. Larval mortality was checked after 24 h of incubation. Each treatment was performed in triplicate. In all the assays, mortality of larvae was recorded and calculated by Abbott formula[14]. The larvicidal activity of Bt SV2 in combination with natural products was subjected to probit regression analysis. The lethal concentrations at 50 % (LC_{50}) were calculated in ppm. Combined formulation of sub lethal dose means $LC_{50/10}$ concentration of Bt SV2 and natural products were mixed 1:1 and evaluated for its larvicidal potential.

Calculations of potency of preparations

The potency of each individual chemical namely Bt SV2, curcumin, plumbagin, camphor, rutin, quercetin, karanjin, pongamol, and their combinations with Bt SV2 was calculated by following formula:

Potency of lab isolate (IU/mg)

$$= \frac{\text{Lethal concentration at 50 \% of Bti}}{\text{Lethal concentration at 50 \% of Bt SV2}} \times 18000$$

Potency of each combination (IU/mg)

$$= \frac{\text{Lethal concentration at 50\% of Bt SV2}}{\text{Lethal concentration at 50\% of test formulation}} \times 18000$$

Fold increase in activity was calculated by following formula:

$$= \frac{\text{Lethal concentration at 50\% individual treatment}}{\text{Lethal concentration at 50\% of combinational treatment}} \times 18000$$

Results and Discussion

Before evaluating the larvicidal potential of selected natural products, they were all tested for their compatibility with Bt SV2. After that, the individual nutrient agar plates were fortified with curcumin, plumbagin, camphor, rutin, quercetin, karanjin, pongamol and were streaked with Bt SV2. It was observed that Bt SV2 showed luxurious growth on the natural products containing nutrient agar plates (Plate. 1). The results indicated that all selected natural products were compatible for formulations with Bt SV2, for action against *Ae. aegypti.*

These natural products were further tested for their individual mosquito larvicidal potential against the fourth instar larvae of *Ae. aegypti*. Rutin, quercetin, and curcumin showed high LC_{50} values (> 100), while karanjin showed significant mortality at comparatively low dose (LC_{50} - 44.59 ppm). Similarly, pongamol, plumbagin, and camphor gave comparable LC_{50} values of 61.18, 59.23, and 71.59 ppm, respectively (Table 1). Standard Bti and lab isolate Bt SV2 were also observed for their larvicidal potential (Table 1).

On the basis of primary larvicidal assay curcumin, plumbagin, camphor, rutin, quercetin, karanjin, and pongamol at sub-lethal concentrations ($LC_{50/10}$) were selected for combinational studies with Bt SV2 in 1:1 proportion. Results indicate that karanjin and plumbagin were the best enhancers of Bt SV2 activity against *Ae. aegypti* with similar LC_{50} values of 1.16 ppm. Plumbagin and karanjin not only showed 1.63 and 1.61 fold increase in Bt SV2 activity but also showed reduction in mortality time. As a result, the original potency of Bt SV2 was increased from 1563.27 to 2567.0 IU/mg by plumbagin.

Plate 1—Representative plate showing luxuriant growth of Bt SV2 on Plumbagin containing plate

This increase in larvicidal potential of Bt SV2 and karanjin, plumbagin may be due to their synergistic effect with Bt SV2[10]. These findings may be supported by earlier studies that indicate insecticidal, insect repellent, and anti oviposition properties of pongam oil[15,16]. Similarly, karanjin also has pesticidal properties[17]. Pongam oil has been reported to show its synergistic effect with some pyrethrins[18,19]. Various components of *P. pinnata* including karanjin have previously been presented as potent antifeedant, insect repellant, and having insecticidal properties against crop pest and mosquitoes[16,20,21]. Plumbagin has also been reported for its mosquito larvicidal potential and it shows effect on growth and development of red cotton bug *Dydercus singulatusi*[22]. Other natural products including curcumin, camphor, and pongamol significantly enhanced the Bt SV2 activity against the *Ae. aegypti* (Table 2). These results were similar with previous reports indicating curcumin, camphor, and pongamol to have mosquito larvicidal and antifeedant effects[15,23,24]. Although rutin and quercetin have been reported to have various biological activities, they didn't show any significant result in combination with Bt SV2 against *Ae. aegypti* larvae and individual preparations of both have very less larvicidal potential.

Table 1—Larvicidal activity of microorganisms and natural products represented in terms of LC_{50} (ppm)

Treatment	LC_{50}	LC_{90}	Mortality Time (h)	Sub lethal dose ($LC_{50/10}$)
Bti (Standard)	1.635	3.88	24	-----
Bt SV2 (Isolate)	1.891`	6.19	24	0.18
Curcumin	118.63	303.19	24	11.8
Camphor	71.59	173.57	20	7.15
Rutin	1096.94	2003.08	24	109.69
Quercetin	770.67	1418.73	24	77.06
Pongamol	61.18	234.08	24	6.11
Karanjin	44.59	341.017	24	4.45
Plumbagin	59.23	264.64	24	5.92

Table 2—Larvicidal activity of combination of Bt SV2 and natural products at 1:1 proportion at sub lethal concentration (LC_{50}/10)

Treatment	LC_{50}	LC_{90}	Mortality Time (h)	IU/mg	Fold increase
Bti (Standard)	1.63	3.88	24	18,000	NA
Bt SV2 (Isolate)	1.891	3.95	24	15563.27	NA
Curcumin	1.31	3.45	12	21,139.58	1.44
Rutin	2.29	2.78	24	22432.89	0.82
Quercetin	2.19	3.44	24	16985.45	0.86
Pongamol	1.33	2.98	12	22,028.44	1.42
Karanjin	1.17	3.12	10	25,171.06	1.61
Plumbagin	1.16	3.02	15	25,670.59	1.63
Camphor	1.76	3.61	12	17369.5	1.07

Conclusion

Environmental unstability of Bt proteins is one of the major hurdle in pest control programs and therefore, potentiation of Bt SV2 is a need of time to fight against the development of pesticide resistance. Use of natural products like curcumin, pongamol, camphor, and karanjin were found to be significant for Bt potentiation. The combinations of Bt SV2 with these natural products may play an important role in delaying pesticide resistance of *Aedes aegypti* owing to decrease in required dose of Bt SV2 and possibly will have different mechanism of action of natural products against mosquito larvae. Alongside of these studies, it is also essential to look at the safety aspects of these natural products against non-target organisms and comprehensive studies are needed for investigation of their actual mechanisms of action.

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