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Mosquito larvicidal and pupaecidal potential of prodigiosin from *Serratia marcescens* and understanding its mechanism of action

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ABSTRACT

Mosquitoes spread lethal diseases like malaria and dengue fever to humans. Considering mosquito vector control as one of the best alternatives to reduce new infections, here we have analyzed the effect of purified pigment prodigiosin extracted from *Serratia marcescens* (NMCC 75) against larval and pupal stages of *Aedes aegypti* and *Anopheles stephensi* mosquitoes. Mosquito larvicidal activities of purified prodigiosin revealed LC_{50} values of 14 ± 1.2, 15.6 ± 1.48, 18 ± 1.3, 21 ± 0.87 µg/ml against early IInd, IIIrd, IVth instar and pupal stages of *Ae. aegypti*, respectively. LC_{50} values for *An. stephensi* were found to be 19.7 ± 1.12, 24.7 ± 1.47, 26.6 ± 1.67, 32.2 ± 1.79 µg/ml against early IInd, IIIrd, IVth instar and pupae of *An. stephensi*, respectively. Further investigations toward understanding modes of action revealed variations in the activities of esterases, acetylcholine esterases, phosphatases, proteases and total proteins in the fourth instar larvae of *Ae. aegypti* indicating intrinsic difference in biochemical features due to prodigiosin treatment. Although there was no inhibition of enzymes like catalase and oxidase but may have profound inhibitory effect on carbonic anhydrase or H^+-V-ATPase which is indicated by change in the pH of midgut and caeca of mosquito larvae. This reduced pH may be possibly due to the proton pump inhibitory activity of prodigiosin. Pure prodigiosin can prove to be an important molecule for mosquito control at larval and pupal stages of *Ae. aegypti* and *An. stephensi*. This is the first report on the mosquito pupaecidal activity of prodigiosin and its possible mechanism of action.

1. Introduction

Vector-borne diseases account for more than 17% of all infections, causing more than 1 million deaths annually. *Anopheles stephensi* and *Aedes aegypti* are the most important vectors to spread deadly diseases like malaria and dengue. More than 2.5 billion people in over 100 countries are at risk of contracting dengue alone. The annual global death toll due to malaria is more than 0.6 million comprising major proportion of children under the age of 5 years [1]. Besides the development of antiparasitic drugs, WHO has considered mosquito vector control as one of the important measures to control diseases caused by mosquitoes. Use of chemical insecticides such as temophos, malathion, and diflubenzuron is the effective method of choice. However, their side effects on human health, environment and development of resistance in mosquitoes against pesticides are causes of concern [2]. Besides this, limited drug availability and development of drug resistance in parasites necessitate the search for new vector control measures.

Natural larvicides include botanical extracts [3], larvicidal oils [4], cyclotides [5], microbial agents like *Bacillus* spp., or their secondary metabolites like cyclic lipopeptides [6], pigments. Similarly, the pupaecidal activity of crude plant extracts against malarial vectors has been reported by few workers [7–11]. Plants have some limitations like unavailability throughout the year, seasonal variations and dependence of their efficacy on the action of multiple metabolites. Microbes do not have these limitations, therefore microbial source of insecticidal metabolites can serve as good alternatives. Microbes and microbial secondary metabolites have been investigated for variety of biological activities. Some bacteria produce pigments as secondary metabolites, which are known to show biological activities, and these include pigments which have multiple ecophysiological roles and activities [12].

Serratia marcescens, a Gram negative, rod shaped bacteria produces red colored pigment prodigiosin which exhibits biological properties such as antibacterial, antifungal, antimalarial, nematicidal, immunosuppressive, and anticancer [13–17]. The mosquito larvicidal effect of the crude prodigiosin was investigated in our previous studies [18]. However, the effectiveness of purified prodigiosin on different larval and pupal stages of mosquitoes has not been investigated yet. Crude extract of prodigiosin from *S. marcescens* contains different metabolites and enzymes like peptidases. It is not

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known whether biological activities of crude prodigiosin are due to pure pigment or enzymes. Therefore, it is necessary to purify and characterize the pigment and to investigate its potential to show biological activities. The mechanism behind the efficacy of prodigiosin in the mosquito larval system is not investigated yet. The effect on enzyme levels in the larval system after treatment with prodigiosin will give an exact understanding about the mechanism of its action.

In the present study, we investigated the (i) effectiveness of pure prodigiosin for larvicidal property against mosquito species, *Ae. aegypti* and *An. stephensi*; (ii) effect of pure prodigiosin on pupal stages of mosquitoes and (iii) mechanism of action of prodigiosin on mosquito larvae.

2. Materials and methods

2.1. Maintenance of larvae

Mosquito larvae were maintained as per procedure by Patil et al. [7]. *Ae. aegypti* and *An. stephensi* eggs were obtained from District Malaria Control Department, Jalgaon (21°2′54″N, 76°32′3″E; elevation, 209 m). The larvae were maintained in dechlorinated tap water. They were fed with a diet of finely ground brewer's yeast and dog biscuits (3:1). The feeding was continued to obtain larvae at early IInd, IIIrd and IVth instars and pupal development stages. These different developmental stages of mosquito larvae were used for the assays.

2.2. Production, purification and identification of prodigiosin

A 24-hour active culture of *S. marcescens* (NMCC 75) was inoculated in a medium containing sucrose and peptone as carbon and nitrogen sources, respectively. The culture was kept at shaking conditions for 24 h at 28 °C ± 2. Pigmented culture was centrifuged at 7000 × *g* for 10 min to get cell mass. Cell pellet was dispensed in methanol and again centrifuged. The crude pigment was recovered in the form of supernatant. The supernatant was further heated at 90 °C so as to get rid of the protein part of the extract. With the heating protein gets precipitated while the dried pigment was redissolved in methanol (2 ml) and added to sterile distilled water (5 ml). After 5–6 hours, the precipitated pigment was layered on a column of silica gel. Partially purified pigment was allowed to run in the column using hexane:methanol (1:2) as a solvent system. Fraction of purified pigment was collected and the pigment was quantified on a dry weight basis.

The absorbance of the pigment was monitored on UV–Visible spectrophotometer (Shimadzu, Japan). FTIR spectra were recorded on Fourier transform infrared spectroscopy (Testscan Shimadzu FTIR 8400, Shimadzu, Japan) and HPLC was performed using Nucleosil C18 column on high-performance liquid chromatography (HPLC) Shimadzu instrument (Kyoto, Japan). The prodigiosin pigment was eluted using a mixture of methanol:water (9:1). Flow rate was kept as 1 ml/min. Detection wavelength for prodigiosin was 536 nm. MS–MS analysis of the prodigiosin was carried out using LCMS-8040 (Shimadzu, Japan).

2.3. Larvicidal bioassay

The larvicidal activity was assessed as per procedure by WHO with some modifications [19]. Experiments were carried out at 28 ± 2 °C and 75–85% relative humidity under 14:10 light and dark cycles. The bioassays were carried out on early IInd, IIIrd and IVth instar larvae of *Ae. aegypti* and *An. stephensi* independently. 25 numbers of larvae of each stage were introduced in a beaker containing 500 ml of dechlorinated tap water. In initial screening 1 mg/ml concentration of pure prodigiosin extracted from *S. marcescens* was assayed. From 6 to 24 hours, the larvae were observed for any movement. Larvae were observed under insect microscope (Labomed) and considered as dead when they didn't show any movement even after pin press.

2.4. 'Dose response bioassay'

Prodigiosin was further bio-assayed using different concentrations against larval and pupal stages of *Ae. aegypti* and *An. stephensi*. Different concentrations ranging from 62.5 to 3.9 μg/ml were prepared from the stalk solutions of the purified pigment. Concentrations of prodigiosin were added in 100 ml dechlorinated tap water, which was further added with 25 larvae of each stage in separate containers. The same quantity of ethanol was used as a negative control. Larval mortality was evaluated at an interval of 6 hours till 24 hours of incubation. Assay was performed in quadruplicates. LC_{50} (50% larval mortality) and LC_{90} (90% larval mortality) values of the pigment concentration were calculated by probit analysis using Minitab software.

2.5. Pupaecidal activity

In a similar way 25 numbers of pupae were assayed with different concentrations (ranging from 62.5 to 3.9 μg/ml) of prodigiosin. In case of pupaecidal activity, the pupae were observed from six hours till 48 hours of incubation under insect microscope for reporting concentration dependent action of microbial pigment on pupae of *Ae. aegypti* and *An. stephensi*. The mature mosquitoes were counted visually. The LC_{50} (50% pupae mortality) and LC_{90} (90% pupae mortality) values of the pigment concentrations were calculated by probit analysis using Minitab software.

2.6. Investigation for mode of action of prodigiosin for mosquito larvicidal potential

We investigated the action of prodigiosin on the functioning of essential enzymes involved in the metabolism of mosquito larvae. The enzymes studied were catalase, esterase, alkaline and acid phosphatase, protease, carbonic anhydrase, general oxidase. All experiments were carried with IVth instar larvae.

2.6.1. Preparation of 'whole body homogenates'

Physiological and biochemical changes were analyzed by biochemical experiments using the procedures outlined by WHO [20]. The prodigiosin treated at LC_{30} concentration (test) and untreated (control) larvae (10 individuals), after 12 hrs, were washed with distilled water and their body surface was blotted with tissue paper to remove the adhering water. The larvae then pooled and homogenized in Eppendorf tubes (held in ice) using a Teflon hand homogenizer in 1 ml of 0.9% w/v saline for eventual estimation of total protein, esterase, acetylcholine esterase, protease and phosphatase assay. Homogenates were centrifuged at 3500 rpm for 10 min at 2 °C in a refrigerated centrifuge. The clear supernatants were kept at 4 °C until use for biochemical analysis.

2.6.2. Protein estimation

The proteins in the larval homogenates of both test and control were first precipitated by 80% ethanol [21] and the protein concentrations were estimated by the method of Lowry et al. [22].

2.6.3. Esterase assay

Carboxyl esterase activity in the larval homogenates was measured by the method of Van Asperen [23] with modifications. Briefly, 200 μl of control and test larval homogenate were mixed with 2 ml of the alpha and beta naphthyl acetate solution, reaction was allowed for 30 min at room temperature. After incubation, 500 μl of the fast