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Potential mode of action of a novel plumbagin as a mosquito repellent against the malarial vector *Anopheles stephensi*, (Culicidae: Diptera)

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ABSTRACT

Plumbagin was isolated and characterized from the roots of *Plumbago zeylanica* using chromatography: TLC, Column chromatogram, HPLC, FTIR and ^{1}H NMR. The isolated pure compounds were assayed for potency as inhibitors of: acetylcholine esterase (AchE), glutathione S-transferases (GST), superoxide dismutase (SOD), cytochrome P450 and α, β-esterase, and for repellency with *Anopheles stephensi* at four different concentrations (25, 50, 75 and 100 ppm). The enzyme assay against the pure compound reveals that the level of esterase and SOD was decreased significantly in contrast the level of GST and cytochrome P450 was increased significantly. Our results suggests that novel Plumbagin has significantly alters the level of enzyme comparable to the control. Evaluations resulted in Plumbagin producing maximum repellency scores against *An. stephensi* mosquitoes in dose dependent manner with highest repellence was observed in the 100 ppm. Histological examination showed that the midgut, hindgut and muscles are the most affected tissues. These tissues affected with major changes including separation and collapse of epithelial layer and cellular vacuolization. The results support the utility of plant compound Plumbagin for vector control as an alternative to synthetic insecticides, however, more vigorous field trials are needed to determine viability under natural conditions.

1. Introduction

The regions of South East Asia reports 70% malarial disease with 200 million cases in India and which are widespread in tropical and subtropical countries [1]. Out of fifty eight species of *Anopheles* six complicated vectors of malarial disease were predominant in India [2]. In these species, *Anopheles stephensi* is a key malaria vector in the Indian subcontinent [3]. Fast growth and development in this region has led to increase in the mosquito population resulting in frequent malaria epidemics [4]. *Plasmodium falciparum* Welch major agent causing malaria primary responsible for illness and mortality in mankind across tropics and subtropics [5]. Mosquito bites may also cause sensitive responses including local skin irritation and systemic reactions such as urticaria and angioedema [6]. It is a lethal sickness resulting in 216 million cases and about 655,000 deaths in 2010 [7]. In India, *An. stephensi* is responsible for malaria transmission in urban areas [8]. Epidemiological results revealed eastern part of India especially Orissa has large number of cases and mortality due to malaria [9].

The over use of these chemical insecticides is not safer due to environment hazard and loss of nontarget organisms, with pest species developing insecticide resistance [10]. Phytochemicals are nowadays considered as preferred alternatives to hazardous and non-biodegradable synthetic pesticides [11–13]. Plant derived compounds are having potential effects against the phytophagous and sometimes haematophagous insects by repellents, anti-feedants and control development [14]. Essential oils derived from the plants act as a good repellent across the countries against haematophagous insects and safeguard the human has been reported earlier [15,16]. Plant derived compounds are familiar, easily degradable, eco-friendly and less toxic in mammals [17] and used in the traditional medicines in most tropical areas [18].

Plumbagin a vital compound, identified from the roots of plant *Plumbago zeylanica* and belongs to one of the major and diverse groups of

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plant metabolites [19–21]. The root of *P. zeylanica* L. has the primary source of Plumbagin. The root part of *P. zeylanica*, is used for treatment of different ailments such as parasitic diseases, fever or malaria [22,23]. The antimalarial activities of Plumbagin have been observed in *An. stephensi* [24]. Numerous studies have focused on pesticidal and mosquitocidal properties of botanicals controlling *Anopheles* mosquito, but have reported different results [25–30].

When an animal deviates from the source due to its odor caused by any compounds then it can be recognized as spatial repellent [31]. In the past few years spatial repellency has been focused more due to their role in controlling the hosts and vectors encounters [32]. The crude volatile oil of plants with their blends of active compounds are used in the commercial repellent formulations against numerous hematophagous insects [33,34]. Coelho et al. [35] investigated the activity of *Moringa oleifera* seed extracts and WSMoL on *Aedes aegypti* larval development and mortality.

Furthermore, acetylcholine (Ach) is a major neurotransmitter which functions as a neuromodulator, modulating nerve impulses transmitted from nerve cell [36]. The neurotransmitter acetylcholine breaks down at the gap between the two nerve cells called synaptic cleft by the enzyme Ach-esterase for allowing the transmission of nerve impulses through the synaptic gap [37]. The mosquito vectors developed resistance towards temephos has been recognized to carrying genes that encode acetylcholinesterase for the selection which afford insensitivity to the chemical pesticides. Insects also express different forms of detoxifying enzymes (glutathione S-transferases-GST, superoxide dismutase-SOD, and esterases) with increased activity [38,39]. Suitable compounds can be used as synergists for insecticides routinely used in control programs [40,41]. But, there is certainly not having any previous evidence regarding a direct bond between enzyme inhibition and repellency in particularly Plumbagin compound in *An. stephensi*. Converging evidence from several fields suggests that many plant based compounds, particularly those concentrated in exposed outsides such as the skins of fruits or roots, function to repel insects and other organisms [42]. The present study investigated the isolation and identification of antimalarial compounds from Plumbagin chemical extractions from *P. zeylanica*. These extractions were then examined for mode of action in repellency, histological changes and inhibitory effects on acetylcholinesterase, glutathione S-transferases (GST), superoxide dismutase (SOD), cytochrome P450 and α- and β-esterases enzyme activities of the malarial vector *An. stephensi*.

2. Materials and methods

2.1. Insects rearing and plant material extraction

The mosquito, *An. stephensi* was reared at the Insectary. Colonies were reared in 30 × 30 × 30 cm cages in rooms maintained at (28 ± 2) °C, 70–85% relative humidity (RH), with a photo period of 14:10 L:D. Food pellets of guinea pig were used for rearing the larvae and 10% sucrose solution were fed to the adults and week old chick for the blood meal.

P. zeylanica roots were collected from natural social forests of Kalakad Mundanthurai Tiger Reserve Forest, Tirunelveli District, India. Roots were dried in shade, and then mechanically pulverized. Then, methanol solvent (60–80 °C, analytical grade, SD fine chemicals) was used for extraction in a soxhlet apparatus until exhaustion. A yellowish orange pastry viscous extract was succumbed. Methanol solvent from the crude was detached in a vacuum rotary evaporator under condensed pressure of 24–28 mm Hg at 38 °C. Further, concentrate was evaporated to dry completely at room temperature. Dry concentrate was stocked in a brown bottle at 4 °C to protect from light.

2.2. TLC and HPLC conformation of bioactive compound

The methanol extract of thin layer chromatography (TLC) was achieved using silica gel (Merk chemicals, India.) with the mobile phase methanol: chloroform (2:4) [43]. TLC was examined at wavelength of 255 and 365 nm under UV light. Column chromatography (240–400 mesh, silica gel) was used with 20 g of methanol extract sequentially eluted with a stepwise mixed slope of chloroform: methanol (8:1, 4:1, 1:1, 0:1) based on increasing polarity. 100 ml of elutents were used and fractions of 50 ml were collected and further thin layer chromatography (TLC) was monitored. Similar fractions were combined to give three fractions 1–4 (a), 5–7 (b) and 8–11 (c). Fraction (c) was further subjected to column chromatography (3.5 cm ID) backed with 40 g silica-gel of mesh size 60–120, and eluted with a chloroform/methanol mixture of increasing polarity as: 8:1, 4:1, 1:1, 0:1. Fraction (c) of 25 ml was collected and TLC monitored using chloroform/methanol mixture (4:2) as mobile phase and this fraction, which exhibited larvicidal efficacy, were examined for purification. Fractions (c), with minimum dose shown high percentage of mortality were further washed with a dilute base of bicarbonate then with distilled water frequently followed by neutralization at every step. Further, the active fraction(c) of methanolic root extract *P. zeylanica* was purified through High Performance Liquid Chromatography (HPLC).

2.3. High Performance Liquid Chromatography

Analysis of compound was carried out through RP-HPLC by using Shimadzu-Promience PDA Chromatogram with reverse-phase column- C_{18} (254 × 4 mm) filled with 5–6 μm diameter particles with UV detector. The temperature was set at 40 °C by using column oven [CTO 10 AS_{VP}]. The HPLC system contained of SIL-6A automatic injector equipped with the 20 μl loop, LC-10 AVP series pumping system, and SPD-10AVP UV detector at 270–280 nm. It was carried out with the mobile phase methanol: acetonitrile: water in the ratio 25:35:40. Shimadzu class VP series software was used to integrate the data and results were compared with standards.

2.4. Identification of Plumbagin

Isolated toxic compound were analyzed Fourier Transform Infrared Spectroscopy (FTIR) and Proton nuclear magnetic resonance (^{1}H NMR). Infrared spectra were confirmed on FT-IR, MAKE- BRUKER Optika GmbH 7500 spectrophotometer in KBr disc. UV spectral data were taken from Hitachi UV-3900 spectrophotometer. ^{1}H was documented on a NMR spectrometer (Bruker Advance DPX-400 MHz) in $CDC1_3$ using TMS at 400 and 100 MHz, respectively. All the chemicals and solvents were used for analytical grade.

2.5. Repellent activity

Repellency studies followed the method of World Health Organization [43]. Blood-starved female (3 d old), *An. stephensi* mosquitoes (100 numbers) were kept in an insect cage (45 cm × 30 cm × 45 cm). The volunteer had no contact with any other chemicals on the experimental day. The volunteer arms, only 25 cm^2 dorsal side of the skin on arms were uncovered and the remaining part covered with rubber gloves. The Plumbagin was used at 25, 50, 75 and 100 ppm/cm^2 individually in the uncovered part of the forearm. Methanol as gave as the control. *An. stephensi* were examined in the night from 19.00 to 05.00 h. The introduced control and treated arm were presented at the same time into the experimental cage and mildly tapping the sides on the mosquito cages, the mosquitoes were activated. Each treatment concentration was repetitive five times. For each concentration the volunteer introduced the treated and control arm into the cages for 1 min simultaneously for every 5 min. The hands were shaken before the mosquitoes suck any blood and the landing time were recorded. The percentage of repellency was calculated by the following formula.

$$\% \text{ Repellency} = [(Ta - Tb)/Ta] \times 100$$