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Toxicity of aristolochic acids isolated from *Aristolochia indica* Linn (Aristolochiaceae) against the malarial vector *Anopheles stephensi* Liston (Diptera: Culicidae)

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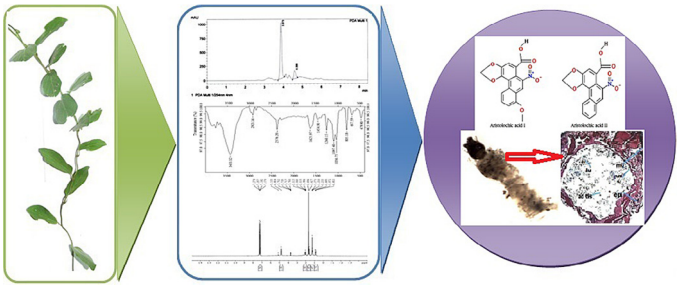
HIGHLIGHTS

- *Aristolochia indica* leaves fractions were identified and estimated for their larvicidal properties against the *A. stephensi*.
- Effective phytocomponents were purified, and functional groups were identified through HPLC and FTIR.
- Bioactive compounds were further confirmed through ^{1}H and ^{13}C NMR.
- Aristolochic acid I and II were proved as a larvicidal agent against the malarial vector *A. stephensi*.
- The greater damage to the gut epithelial cells was observed through histology.

GRAPHICAL ABSTRACT



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ABSTRACT

With the growth of resistance to overused insecticides, vector management has become highly problematic. Hence more concentration has been focused on botanicals. Therefore our present study was aimed to evaluate the toxicity of compounds, aristolochic acid I and aristolochic acid II from the methanol extract of *Aristolochia indica* L. (Aristolochiaceae) leaves on larvae of *Anopheles stephensi* L. (Diptera: Culicidae) employing World Health Organization standard larvicide testing procedures. The soxhlet extraction was carried out using polar solvent, methanol. The isolated toxic compounds were purified through RP-HPLC. The FTIR spectroscopic studies revealed different peak values with functional groups in the mixed compounds (AA-I and AA- II). These two aristolochic acids were further studied through ^{13}C and $^{1}HNMR$ analysis with confirmed by structures. Bioassay-guided fractionation through flash chromatography lead to the isolation of two larvicidal compounds namely aristolochic acid I and II. In these bioassays, the larvae were exposed to concentrations of 100, 250, 500,750 and 1000 ppm for each compound. Between the two, AA-I exerted no significant toxicity difference ($P < 0.05$) on mosquito larvae with LC_{50}- 171.3, 209.8, 269.1, 502.3 ppm and LC_{90}-751.6, 963.8, 972.7, 990.8 ppm compared to AA-II with LC_{50}-134.8, 166.7, 240.4,

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543.2 ppm and LC_{90}- 636.7, 792.5, 990.8, 986.2 ppm against first, second, third and fourth instars, respectively. Further, the isolated compounds were severely affecting the mosquito gut. From the results, *A. indica* toxic compounds could be considered as one of the influential applicant to bring about useful botanicals so as to prevent the resurrection of mosquito vectors.

1. Introduction

Mosquitoes are vectors of etiologic agents of malaria, filariasis and viral diseases. Malaria is a deadly disease that resulted in 207 million cases and about 627,000 deaths in 2012 (WHO, 2013). *Anopheles stephensi* Liston (Diptera: Culicidae) is the primary vector of malaria in India and other West Asian countries and improved methods of control are urgently needed (Burfield and Reekie, 2005; Senthil-Nathan et al., 2005a, 2008). In India, *A. stephensi* is responsible for malaria transmission in urban areas (Dash et al., 2008). The continuous use of synthetic insecticides causes side effects on non-target organisms and insecticide resistance in mosquitoes (Kelm et al., 1997). The constant use of chemical insecticides leads to the interruption of natural biological control systems and outbreaks of insect species (Milam et al., 2000). This constant use of synthetic insecticides also leads to insect resistance, environmental pollution and undesirable effects on humans, mammals, and other non-target organisms (Brown, 1986).

Although, effective drugs were available to kill these resistant parasites, they are not readily available or affordable in many developing countries (Wilcox and Gilbert, 2012). The insecticides weaken the cuticle defense system of the larvae causing penetration of pathogenic organisms, thus reducing the mosquito population (Batabyal et al., 2007; Dua et al., 2009). This has necessitated requiring for search and development of environmental secure, biodegradable and indigenous method for vector control (Amer and Mehlhorn, 2006). On the other hand, (WHO, 2013) classifies insecticide resistance status based on bioassay mortality (resistant if mortality rate is <90%, possible resistance if mortality is between 90- 97%, and susceptible if the rate is >98%).

Recently, there has been a main concern for the endorsement of botanicals as environmentally friendly pesticides, microbial sprays, and insect growth regulators amidst other control measures such as beneficial insects and all, require an addition of supervised control (Ascher et al., 1995; Senthil-Nathan et al., 2004, 2006). A quantity of reports establish the mosquito larvicidal potential of the plant extracts and the essential oils obtained from the different parts of the variety of plants (Kalaivani et al., 2012; Senthil-Nathan, 2007, 2013, 2015; Thanigaivel et al., 2012), though the insecticidal effects of plant chemicals differ not only according to plant species, mosquito species and plant parts, but also to extraction methods. Global trade in plant-based drugs was estimated at US$ 100 billion, of which traditional medicines using medicinal plants accounted for 60 billion (WHO, 2004). Several studies have targeted on insecticidal, larvicidal, and repellent properties of natural products for controlling *Anopheles* mosquito with different results (Ansari et al., 2000; Muthukrishnan and Pushpalatha, 2001; Prajapati et al., 2005; Pushpalatha and Muthukrishnan, 1999; Saxena et al., 1993). Many studies on plant extracts against mosquito vectors have been carried out around the world, but most of them are limited to preliminary screening (Chaiyasit et al., 2006; Promsiri et al., 2006).

Aristolochia indica (Linn) *Aristolochiaceae* is a native of India and is commonly named as Iswar mul and perennial climber with greenish white woody stems found throughout India in the plains and low hills. *Aristolochiaceae* plant family contains a variety of compounds which have shown insecticidal, growth regulating and development modifying properties (Marlin and Rajeshkumar, 2012). Antifeedant activity of metabolites of *Aristolochia albida* against the tobacco cutworm, *Spodoptera litura* was observed (Lajide et al., 1993).

The crude methanol, hexane and ethyl acetate extracts of *A. indica* were effective for larvicidal, adulticidal and repellent activity against adult and early fourth-instar larvae of *Culex gelidus* and *Culex quinquefasciatus* (Kamaraj et al., 2010) but no reports are available on the larvicidal property of the bioactive compounds.

Aristolochic acids are compounds of nitrophenanthrene carboxylic acids that occur naturally in plants of the family *Aristolochiaceae*, primarily in the genera *Aristolochia* and *Asarum* (Huang et al., 2005; Hsieh et al., 2006). A new naphthoquinone Aristolindiquinone (Che et al., 1983), Aristolochic acids and Aristolactams (Mix et al., 1982) was reported from *A. indica*. The purpose of the present investigation was designed to identify, isolate and to test the toxic compounds from *A. indica* leaves against *A. stephensi.*

2. Materials and methods

2.1. Extraction of plant material

A. indica leaves were collected from trees of natural forests of Kalakadu Mundanthurai Tiger Reserve Forest, Tirunelveli district, India and shade dried. These were mechanically pulverized and subjected to extraction in a soxhlet apparatus using methanol solvent (60–80 °C, AR grade obtained from SD fine chemicals, Bombay) until exhaustion, which yielded an yellowish brown viscous pastry mass (extract). Solvent from the extract was detached in a vacuum rotary evaporator under reduced pressure of 22–26 mmHg at 40 °C and concentrate was further evaporated to complete dryness at room temperature. This was stored at 4 °C in a brown bottle and assayed for its larvicidal efficacy using standard procedures (WHO, 2005).

2.2. Isolation and purification of bioactive compounds

Thin layer chromatography (TLC) of the methanol extract was performed using silica gel (Merk chemical Ltd.) with the mobile phase benzene: acetone: methanol (6:3:1). Chromatogram was examined under UV light at wavelength of 254 and 366 nm. Flash column chromatography (silica gel 240–400 mesh) was carried out by extracting 20 g of formulation in methanol and successively eluting with a stepwise gradient of ethyl acetate: hexane (0:100; 10:90; 20:80, 30:70,40:60, 50:50). Fifty ml of subfraction in 40 test tubes were collected. They were monitored by TLC and pooled so as to give four major fractions namely F1, F2, F3 and F4. Fractions were evaporated at room temperature to attain thick phytocomponents and verified for larvicidal property. Fractions, which showed larvicidal efficacy, were subjected to purification. Two fractions (F3 and F4), which gave high percent mortality in minimum dose were subsequently washed with bicarbonate, a dilute base, an acid and with distilled water repeatedly followed by neutralization at each step. Toxic compounds from these fractions were isolated and crystallized, passing over activated charcoal with hot ethanol to take out impurities if any. Purity of the compounds was confirmed through RP-HPLC by using Shimadzu-Promience PDA Chromatogram with C_{18} reverse-phase column (254 × 4 mm) with UV detector. Phytocomponents free from impurity were carefully evaporated to complete dryness at room temperature and