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Chemical characterization of n-alkane compounds in the leaves of *Holoptelea integrifolia* and its repellence against Japanese encephalitis vector

S. Singha*[1, 2] and G. Chandra[2]

[1]*Department of Zoology, Vivekananda Mahavidyalaya, Burdwan, West Bengal, India*
[2]*Microbiology and Nanotechnology Research Laboratory, Department of Zoology, The University of Burdwan, West Bengal, India.*
Email: someshbio@gmail.com; goutamchandra63@yahoo.co.in

ABSTRACT: Epicuticular wax extract bearing n-alkane compounds were isolated from leaves of *Holoptelea integrifolia* and its chemical characterization was done by GC-MS analysis. Seven n-alkane compounds were isolated from epicuticular wax of *H. integrifolia,* which are Undecane [$C_{11}H_{24}$], Decane 5-methyl- [$C_{11}H_{24}$], Dodecane [$C_{12}H_{26}$], Undecane, 3,6-dimethyl- [$C_{12}H_{26}$], Hexadecane, 2,6,10,14-tetramethyl- [$C_{20}H_{42}$], Tridecane [$C_{13}H_{28}$], and Tetradecane [$C_{14}H_{30}$]. Different concentrations of crude extract as well as epicuticular wax extract bearing n-alkane each @ 2, 4 and 5 ppm cm^{-2} applied on human hand surface for repellence against *Culex vishnui* (vectors of JE) and at different time of exposure, gave a maximum protection of 73.33 per cent in the case of crude extract, and 94.33 per cent with epicuticular wax extract, both at 5 ppm cm^{-2}, up to five hours of exposure.

KEYWORDS: Indian Elm tree, epicuticular wax extract, repellent, *Culex vishnui* group

Mosquito at the time of blood feeding transmits extremely harmful pathogens from host to host causing malaria, yellow fever, dengue, zika, filariasis, and Japanese encephalitis (JE). Female mosquito uses blood meal as protein and vitamin source for egg development and blood proteins are used as building blocks for the synthesis of egg yolk proteins. The first major epidemic of JE in India was reported from Bankura and Burdwan districts of West Bengal in 1973 (*Curic et al., 2014),* caused by the mosquito borne JE virus (Mahmud *et al*., 2010). According to WHO (1981) more than 3 billion people of South-East Asia and Western Pacific regions are under the risk of JE transmission. Extracts of different parts of several plants have been reported earlier as mosquito repellent along with others activities (Adhikari *et al.,* 2012; Rawani *et al.,* 2012; Adhikari and Chandra*,* 2014; Bhattacharya and Chandra, 2014; Haldar *et al.,* 2014).

Epicuticular wax on the surface of plant leaves and other parts of the plant plays an important ecological role in interaction with insects as attractant or deterrent (Muller, 2006). *Holoptelea integrifolia* (Roxb.) belonging to the family Ulmaceae and commonly known as the Indian Elm tree, is found all over the Indian peninsula (Mahmud *et al.,* 2010). From ancient times, this tree was well known due to its medical importance. Traditionally different parts of this plant were used for the treatment of different diseases like inflammation, gastritis, dyspepsia, colic, intestinal worms, vomiting, wound

* Author for correspondence

healing, leprosy, diabetes, dysmenorrhoea and rheumatism (Kumar *et al.,* 2011). According to Singha *et al.* (2012) acetone extract of leaf of *H. integrifolia* have very good larvicidal activity on *Cx. vishnui* group of mosquitoes. Antibacterial efficacy was also established from different solvent extracts of leaf of *H. integrifolia* against beta-lactam resistant strain of *Staphylococcus aureus* but diethyl ether extract with 1, 4-naphthalenedione as the bioactive principle was found to perform the best result (Vinod *et al.,* 2010). Epicuticular wax of leaf of this plant bears a mixture of different straight or branched chain alkane, esters, aldehydes, alcohols, and fatty acids, among others (Kunst and Samuels, 2003; Koch and Ensikat, 2008), but alkanes play vital role in plant-insect interaction (Müller, 2005; 2006).

Present study was aimed at isolating n-alkane compounds from epicuticular wax of leaf of *H. integrifolia* by application of non-polar solvent (n-hexane) and characterization of n-alkane compounds by GC-MS analysis as well as to establish their role as mosquito repellent on adult *Cx. vishnui* group of mosquitoes.

Fresh and mature leaves of *H. integrifolia* were harvested randomly from plants growing at the outskirts of Dedipur, Burdwan, India. Collected mature leaves were initially rinsed in tap water followed by distilled water to remove dust, unwanted debris etc. and dried on a paper towel. Crude leaf extract was prepared by mortar and pestle and the extract was allowed to become a semisolid paste through simple air drying. Fifty grams of leaves were dipped in two litre of cold n-hexane solution for extraction of epi-cuticular wax at room temperature for 45 min. The extract was filtered through Whatman no.41 filter paper. The filtrate was dried in rotary evaporator. Thin layer chromatography (TLC) was done for fractionation of extract, where carbon tetrachloride was used as mobile phase. Calculated R_f value from TLC was 0.68. The TLC fractions with same Rf value (Rf value = 0.68) were scraped and collected from 40 TLC plates. TLC plates (thickness of 0.5 mm) were prepared with silica gel G (Merck, Mumbai, India) using a Unoplan coating apparatus (Shandon, London, UK) (Bhattacharjee *et al.,* 2010).

Larvae of *Cx. vishnui* group of mosquitoes were collected from rice fields surrounding Golapbag campus, The University of Burdwan. Larvae were reared in plastic tray till they transformed into the pupa. Pupae were isolated manually by dropper and transferred to mosquito cage to metamorphose into adult form. Blood starved adult female mosquitoes were used for the repellence test. Repellence activity of crude extract and epicuticular wax of leaf of *H. integrifolia* was studied separately on human volunteers following WHO (2009) protocol. Required approval was obtained from the Institutional Ethics Committee (IEC/BU/2021/3, dt-24/6/21).

Three to four day old blood starved 100 adult female mosquitoes were kept in a net cage. Repellence test was conducted in a cage measuring 70 x 60 x 30 cm^3. Isopropanol was used for cleaning the arms of the volunteer. After air-drying 25 cm^2 area of the skin surface on each arm was exposed, remaining areas were being covered by rubber gloves. 2 ppm cm^{-2}, 4 ppm cm^{-2} and 5 ppm cm^{-2} concentrations of each of the crude extract and epicuticular wax of leaf of *H. integrifolia* were applied on the exposed area of the experimental arm. On the control arm, respective extracts were not applied before exposing to starved mosquitoes in the cage. The numbers of bites were recorded over 5 minutes after every 60 min, from 0.00 h to 5.00 h. Each experiment was repeated three times. Percentage of protection from mosquito bite was measured by using the following formula:

$$\text{Protection (\%)} = \frac{\text{Number of bites received by control arm - Number of bites receieved by treated arm}}{\text{Number of bites received by control arm}} \text{ X } 100$$

Characterization of epicuticular wax was done by GC-MS analyses, following NIST (National Institute of Standards and Technology) Library. One ìl sample was injected in split mode in the instrument (GCMS-QP2010 plus). During sample injection the initial temperature was set at 60 ^{0}C and the temperature was increased to 270 ^{0}C in a successive manner. In the whole procedure Helium was used as carrier. Mass spectral analysed data

were recorded with 40-650 m/z scanning range and with speed of 5 scan sec^{-1}. Statistical analyses of collected data were done through Microsoft Excel software.

In the repellence test the highest efficacy of protection for 5 hours, was recorded at 5 ppm cm^{-2} with the epicuticular wax extract bearing n-alkane compounds (94.33 %) and in the crude extract (73.33%). The mean repellency potential varied significantly with crude extract and epicuticular wax extract on *Cx. vishnui* group and again with different duration of exposure and at different concentrations (Table 1). Further observations revealed that the potentiality of the crude extract and epicuticular wax extract gradually decreased after five hours and the activity persisted up to 8 h.

Table 1. Repellence of crude extract and epicuticular wax extract on *Culex vishnui* mosquito

Conc. (ppm cm^{-2})	Duration of exposure (h)	Protection (Mean ±SE) (%)	
		Crude	Epicuticular wax
2	3	37.67±0.33	64.67±0.67**
4	4	63.33±0.33	82.67±0.67**
5	5	73.33±0.33	94.33±0.33**

** *Highly significant; (Mean ±SE)*

GC-MS analysis of n-alkane compounds isolated and identified were as follows: Undecane [C11H24], Decane 5-methyl- [C11H24], Dodecane [C12H26], Undecane, 3,6-dimethyl- [C12H26], Hexadecane, 2,6,10,14-tetramethyl- [C20H42], Tridecane [C13H28], and Tetradecane [C14H30]. Out of isolated seven compounds, dodecone, undecane and tridecane are present as major constituents in epicuticular wax from leaf of *H. integrifolia*. Of these compounds, only dodecane and tetradecane were previously reported for their mosquito repellence and others were not reported for their mosquito repellence (Pojmanova *et al.,* 2019; El-Sayed, 2020; Lu *et al.,* 2020; Sutthanont *et al.,* 2010). In epicuticular wax of plants, the major components are n-alkanes, which are mainly ranging from C12 to C27 in carbon chain lengths. Epicuticular wax has many physiological functions, including protection against UV light and moderation of gas exchange through stomata (Bhattacharjee et al. 2010). Interaction between plant-insect and plant-pathogen were mainly triggered by epicuticular wax components of plant parts (Müller, 2006; Carver and Gurr, 2006). Leaves of different plant species contain different n-alkane profile (Barik et al., 2004; Jetter and Schäffer, 2001).

Isolated epi-cuticular wax from leaf of *H. integrifolia* has repellent potentiality against JE vector, *Cx. vishnui.* It shows repellence at a very low concentration for a longer time. Both crude and epi-cuticular wax bearing n-alkane compounds are safe for use on skin surface, as the application has not shown any discomfort or reaction. It may be a better alternative against commercially available different mosquito repellents.

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