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Larval control of *Culex vishnui* group through bio-active fraction of traveller's tree, *Ravenala madagascariensis* Sonn. (Strelitziaceae)

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Abstract

Background and objectives: Due to ever increasing resistance against synthetic insecticides, mosquito control is fetching a serious problem all over the world. It is imperative to manage the vector population to conquer the mosquito born diseases. The present study was carried out to assess the target specific larvicidal activity of *Ravenala madagascariensis* against *Culex vishnui* group, the vector of Japanese Encephalitis.

Methods: Crude extracts of *R. madagascariensis* mature leaves (foliages) ranging from 0.1% to 1.0% concentrations were tested against all the larval instars of *Cx. vishnui* group. Solvent extractions of mature leaves were carried out through three different solvents viz. petroleum ether, ethyl acetate and acetone from non-polar to polar trend. Larvicidal activities of the active fractions were examined against all the larval instars with graded concentrations ranging from 50 ppm to 250 ppm. LC₅₀ and LC₉₀ values were determined by log-probit analyses. Further statistical justifications were done through ANOVA analyses. Effectiveness of the bioactive fractions against non-target populations was executed in laboratory conditions. Phytochemical screening of leaf extract was also carried out.

Result and Discussion: At 72 hours post-exposure, highest mortality (100%) with crude extract was found at 0.5% concentrations against all the instars. Amongst the three bioactive fractions, ethyl acetate extractives showed the highest larval mortality. After 72 hours of exposure, 200 ppm and 250 ppm concentration showed 100% mortality against 1st and 2nd instars larvae respectively. A 96.00% reduction in 3rd instars mosquito population was recorded after 72 hours at 250 ppm concentration. However, 4th instars larvae were subjected to only 84.00% reduction with these experimental set up and at 250 ppm concentration. The results of log probit analyses (95% confidence level) showed that LC₅₀ and LC₉₀ values were gradually decreased with the exposure periods having the lowest value at 72 h of exposure to 1st instars larvae followed by 2nd, 3rd and 4th instars larvae. Mortality rate (Y) was found to be positively correlated with the concentration (X) having a regression coefficient (R²) close to 1 in each case. Phytochemical analyses revealed the qualitative presence of tannin, steroid and alkaloid free glycoside bound anthraquinones. Non target organisms were non-responsive to the bioactive fractions obtained from the plant throughout the experiment.

Conclusion: From the above experiment it can be concluded that the mature leaves (foliages) of *R. madagascariensis* may be a superior larvicide alternative to the synthetic one.

Keywords Ethyl acetate extract; *Culex vishnui* group; Larvicide; Phytochemical screening

Introduction

Mosquito is a small iniquitous dipteran fly highly responsible for worldwide transmission of some deadly diseases like malaria, filariasis, dengue, encephalitis, chikungunya and various other diseases which cause numerous deaths annually. In tropical country like India, about 40 million people are affected each year with such mosquito born diseases (Ghosh *et al.*, 2012). Amongst the repugnant diseases, Japanese Encephalitis (JE), caused by a flavivirus

from flaviviridae family, is prevalent in South East Asia and Far East. Domestic pigs and some wild birds act as a reservoir of the disease. There are more than 50,000 cases per annum and 15,000 deaths only in Indian sub-continent (Kabilan *et al.*, 2004) and approximately 3 billion populations of the world inhabitants exist in JE-endemic regions. Population of rural areas are at greater risk than urban areas. In tropical area the JE virus has been isolated from various mosquito species, so it can be concluded that

culicine mosquitoes especially *Culex vishnui* group (*Cx. tritaeniorhynchus*, *Cx. vishnui* and *Cx. pseudovishnui*) are the principal vector of JE virus (Colless, 1957, Hasegawa et al., 2008). *Culex vishnui* group is prevalent and preferably breeds in water with plentiful vegetation generally in paddy fields (Banerjee and Chandra, 2004) and it is profusely found in shallow ditches, pools and rice cultivation sites, so, India is a highly endemic country for JE.

Many synthetic insecticides which are present in the market with substantial achievement are detrimental to other non-target organisms, as well as human health (Brown, 1986) and those imply bio-magnifying hazards owing to their non bio-degradability and residual exposure (Wattal et al., 1981). Nowadays the chemical insecticides are found resistant to vector population for its widespread use (WHO, 1992). So, it is important to find a suitable alternative insecticide which is eco-friendly and target specific. Insecticides of botanical origin (Ray et al., 2014; Chowdhury et al., 2009) may play a major role in this field.

Ravenala madagascariensis, commonly known as traveller's tree or traveller's palm, is not a true palm (family-Arecaceae) but a member of the family, Strelitziaceae. It is the sole member of the genus *Ravenala*. The height of plant is about 7 m; the sheath of the enlarged leaves of the plant can hold rain water, but the water is contaminated by organic impurities,

so, it smells bad and tastes awful. Paddle shaped, banana like leaves with long petioles are aligned in a single plane. Flowers are small, creamy white in colour, upto 30 cm long, fruits are 2-4 cm long, woody capsule. For its distinctive habit and foliage, it is extensively cultivated in tropical and subtropical regions. It is known that *Ravenala madagascariensis* is traditionally used in treatments of kidney stone, diabetes (Shakthi et al., 2010) and antiseptic activity (Jain and Srivastava, 2005). According to our literature review there is no such information regarding its use as insecticide. So this is the first ever report that it can be used as potential mosquito larvicide against *Cx. vishnui* group.

1 Result and Discussion:

Significant larvicidal activity of *R. madagascariensis* leaf extractives were clearly noticed under laboratory condition. Mosquito larvicidal potentiality of different concentrations of crude extracts against all the larval instars is shown in Table 1. After 72 hours of exposure 100% mortality was found against 1st instars larva at 0.5% concentration. Higher Mortality rate gradually increased with exposure period for all time instars larvae. Higher mortality was recorded at 0.5% concentration for each instars than other cons. tested. The percent mortality did not increase significantly at higher than 0.5% of crude extracts and those data were excluded.

Table 1 Percent mortality of *Cx. vishnui* group larvae using crude extract of *R. madagascariensis* leaves

| Larval Instars | Concentration (%) | Percent mortality (Mean ± SE) | | |
|----------------|-------------------|-------------------------------|--------------|---------------|
| | | 24 h | 48 h | 72 h |
| First | 0.1 | 30.67 ± 1.25 | 34.67 ± 0.82 | 41.33 ± 0.94 |
| | 0.2 | 38.67 ± 1.63 | 46.67 ± 1.41 | 52.00 ± 0.82 |
| | 0.3 | 49.33 ± 2.05 | 60.00 ± 0.00 | 65.33 ± 0.94 |
| | 0.4 | 65.33 ± 1.63 | 74.67 ± 0.94 | 80.00 ± 1.47 |
| | 0.5 | 70.67 ± 0.80 | 76.00 ± 0.65 | 100.00 ± 0.00 |
| Second | 0.1 | 24.00 ± 0.94 | 32.00 ± 0.82 | 36.00 ± 1.70 |
| | 0.2 | 34.67 ± 2.05 | 40.00 ± 1.63 | 42.67 ± 0.65 |
| | 0.3 | 52.00 ± 2.16 | 56.00 ± 2.05 | 60.00 ± 0.82 |
| | 0.4 | 58.67 ± 1.70 | 68.00 ± 0.82 | 74.67 ± 1.25 |
| | 0.5 | 66.67 ± 1.41 | 73.33 ± 0.47 | 77.33 ± 0.00 |
| Third | 0.1 | 22.67 ± 0.00 | 29.33 ± 0.80 | 36.00 ± 1.47 |
| | 0.2 | 32.00 ± 0.94 | 37.33 ± 1.25 | 41.33 ± 2.45 |
| | 0.3 | 49.33 ± 0.00 | 53.33 ± 1.41 | 57.33 ± 0.00 |
| | 0.4 | 57.33 ± 1.94 | 64.00 ± 1.25 | 72.00 ± 1.41 |
| | 0.5 | 62.67 ± 1.63 | 69.33 ± 0.00 | 74.67 ± 0.00 |
| Fourth | 0.1 | 0.00 ± 0.00 | 8.00 ± 1.25 | 24.00 ± 0.00 |
| | 0.2 | 2.67 ± 1.25 | 9.33 ± 0.47 | 30.67 ± 0.00 |
| | 0.3 | 6.67 ± 0.00 | 13.33 ± 2.87 | 34.67 ± 0.47 |
| | 0.4 | 9.33 ± 0.47 | 17.33 ± 1.45 | 36.00 ± 0.82 |
| | 0.5 | 12.00 ± 1.28 | 25.33 ± 1.63 | 45.33 ± 2.16 |

Larval mortality of all the different instars was presented in Table 2. Amongst the three different solvent extracts ethyl acetate showed positive larvicidal effect. After 72 hours of exposure, 1st instars as well as 2nd instars larvae

exhibited 100% mortality in 200 ppm and 250 ppm concentrations of ethyl acetate extractives respectively. In case of 3rd and 4th instars larvae highest mortality was found in 250 ppm concentration at 72h of exposure.

Table 2 Percent mortality of *Cx. vishnui* group larvae using ethyl acetate extract of *R. madagascariensis* leaves

| Larval Instars | Concentration (ppm) | Mortality rate (Mean \pm SE) | | |
|----------------|---------------------|--------------------------------|------------------|-------------------|
| | | 24h | 48h | 72h |
| First | 50 | 56.00 \pm 0.94 | 68.00 \pm 0.94 | 80.00 \pm 0.82 |
| | 100 | 61.33 \pm 1.63 | 74.67 \pm 2.05 | 86.67 \pm 2.95 |
| | 150 | 76.00 \pm 2.05 | 80.00 \pm 1.70 | 96.00 \pm 0.94 |
| | 200 | 82.67 \pm 1.63 | 86.67 \pm 0.94 | 100.00 \pm 0.00 |
| | 250 | 86.67 \pm 0.80 | 92.00 \pm 0.65 | 100.00 \pm 0.00 |
| Second | 50 | 49.33 \pm 0.80 | 57.33 \pm 0.82 | 68.00 \pm 0.82 |
| | 100 | 54.67 \pm 1.25 | 67.67 \pm 1.63 | 77.33 \pm 2.87 |
| | 150 | 62.67 \pm 2.16 | 72.00 \pm 1.41 | 80.00 \pm 0.82 |
| | 200 | 69.33 \pm 1.25 | 77.33 \pm 0.81 | 94.67 \pm 1.25 |
| Third | 250 | 77.33 \pm 1.41 | 85.33 \pm 0.47 | 100.00 \pm 0.00 |
| | 50 | 36.00 \pm 0.00 | 41.33 \pm 0.47 | 61.33 \pm 1.47 |
| | 100 | 41.33 \pm 0.82 | 58.67 \pm 1.25 | 74.67 \pm 2.45 |
| | 150 | 48.00 \pm 0.00 | 66.67 \pm 1.25 | 80.00 \pm 0.00 |
| | 200 | 66.67 \pm 1.94 | 73.33 \pm 1.25 | 85.33 \pm 0.47 |
| Fourth | 250 | 73.33 \pm 0.94 | 81.33 \pm 0.00 | 96.00 \pm 0.00 |
| | 50 | 33.33 \pm 1.92 | 38.67 \pm 0.00 | 57.33 \pm 0.82 |
| | 100 | 37.33 \pm 0.47 | 53.33 \pm 0.47 | 70.67 \pm 2.47 |
| | 150 | 48.00 \pm 0.00 | 58.67 \pm 1.28 | 76.00 \pm 0.47 |
| | 200 | 64.00 \pm 0.47 | 65.33 \pm 1.45 | 82.67 \pm 0.82 |
| 250 | 69.00 \pm 0.47 | 72.00 \pm 0.47 | 84.00 \pm 0.82 | |

The results of log probit analyses at 95% concentration showed that LC₅₀ and LC₉₀ values progressively reduced with increase in time of exposure. LC₅₀ and LC₉₀ values for 1st instar larvae at 72h of exposure were 25.41 μ g.mL and 90.98 μ g.mL respectively which were significantly low doses.

The results of regression analyses of ethyl acetate extracts of *R. madagascariensis* leaves revealed that the mortality rate (Y) was positively correlated with the concentration (X) having a regression coefficient (R²)

close to 1 in each case (Table 3). The larvicidal role of the plant under study was found to be statistically justified through ANOVA analyses (Table 4). The result of preliminary screening of phytochemicals from *R. Madagascariensis* leaves was given in Table 5. Phytochemicals like tannin, steroid and alkaloid free glycoside-bound anthraquinones were detected from the plant leaves. No mortality or abnormalities related to sluggishness of swimming activity was observed in non-target organism after 72h of exposure.

Table 3 Assessment of LC₅₀ and LC₉₀ values of ethyl acetate extract of *R. madagascariensis* through log-probit and regression analyses

| Larval Instars | Period of Exposure | LC ₅₀ | LC ₉₀ | Regression | R ² - value |
|-----------------|--------------------|------------------|------------------|----------------|------------------------|
| 1 st | 24 | 44.65 | 466.43 | 0.04 x + 11.93 | 0.96 |
| | 48 | 23.49 | 315.52 | 0.03 x + 15.56 | 0.99 |
| | 72 | 25.41 | 90.98 | 0.03x + 19.13 | 0.90 |
| 2 nd | 24 | 69.12 | 1230.15 | 0.04x + 10.36 | 0.99 |
| | 48 | 40.95 | 593.06 | 0.03x + 12.93 | 0.99 |
| | 72 | 32.96 | 175.13 | 0.04 x + 14.89 | 0.96 |
| 3 rd | 24 | 119.23 | 1096.75 | 0.05x + 5.77 | 0.95 |
| | 48 | 77.67 | 566.48 | 0.05x + 8.97 | 0.96 |
| | 72 | 39.07 | 241.46 | 0.04x + 13.86 | 0.97 |
| 4 th | 24 | 133.08 | 1056.58 | 0.05x + 5.20 | 0.96 |
| | 48 | 97.92 | 1026.96 | 0.04x + 8.50 | 0.96 |
| | 72 | 38.46 | 457.29 | 0.03x + 13.63 | 0.91 |

Note: x = concentration (in ppm) of solvent extracts

Table 4 Completely randomized three way ANOVA analyses using concentration (C), hour (H) and instars (I) as three independent parameters

| Source of variation | Sum of squares (SS) | Degree of freedom (df) | Mean of squares (MS) | F value | p-level |
|---------------------|---------------------|------------------------|----------------------|---------|---------|
| Instars (I) | 1079.91 | 3 | 359.97 | 285.44 | 0.00 |
| Hours (H) | 670.08 | 2 | 335.04 | 265.67 | 0.00 |
| Conc. (C) | 1223.02 | 4 | 305.76 | 242.45 | 0.00 |
| I × H | 50.99 | 6 | 8.50 | 6.74 | 0.00 |
| I × C | 31.42 | 12 | 2.62 | 2.08 | 0.02 |
| H × C | 15.31 | 8 | 1.91 | 1.52 | 0.15 |
| I × H × C | 20.51 | 24 | 0.85 | 0.68 | 0.86 |
| Within groups | 151.33 | 120 | 1.26 | --- | --- |
| Total | 3242.57 | 179 | 18.12 | ---- | --- |

Table 5 Qualitative analyses of phytochemicals of crude extract of tested plant leaves

| Name of the Plant | Plant Part | Tannin | Saponin | Steroid | Flavonoid | Terpenoid | Cardiac glycosides | Alkaloid free glycoside-bound Anthraquinones |
|----------------------------------|------------|--------|---------|---------|-----------|-----------|--------------------|--|
| <i>Ravenala madagascariensis</i> | Leaves | ++ | -- | ++ | -- | -- | -- | ++ |

Note: ++ Present __ Absent

Control of the mosquito borne diseases is a challenge nowadays and a great problem throughout the world. Mosquito control mainly depends on control of larvae and adults. Larval control is easier than control of adults due to their confinement to water bodies. Synthetic insecticides exhibit rapid success in the field of insect control than their counterparts of botanical origin. But, in a few decades, due to quick development of resistance against the chemical insecticides, use of biodegradable, cost-effective botanicals has gained importance. Various workers have published their works with natural insecticides having a remarkable larvicidal potentiality (Singha et al., 2012; Bhattacharya and Chandra 2013, 2014; Kundu et al., 2013, Chakraborty et al., 2013). Plant components have been reported as repellent, adulticidal and smoke toxic (Singha et al., 2011, Chowdhury et al., 2007) and pupicidal (Rawani et al., 2012) against different mosquito species. The present study has represented the target specific larvicidal activity of *R. madagascariensis* leaves against *Cx. Vishnui* for the first time. It can be a potential larvicidal agent in very near future.

2 Material Methods:

2.1 Collection of plant material

Fresh leaves of *R. madagascariensis* were collected from the area of Sundarban (21° 56' 59" N, 89°

10' 59.988" E), West Bengal, India. A voucher specimen (GCP-14) was acquiesced to the herbarium of Department of Zoology, The University of Burdwan.

2.2 Processing of crude extract

Collected fresh, mature unspotted green leaves of *R. madagascariensis* were firstly chopped to standard size. After that the chopped leaves were rinsed in tap water followed by distilled water. Then the leaves are soaked on a paper towel. The dirt-free and unspotted leaves were crushed by the mechanical grinder and the fluid was filtered by Whatman's Grade 4 (20 - 25 µm) filter paper. The filtrate was stored as a stock solution (100% concentration) for the further bioassay experiments.

2.3 Solvent extraction

Unsoiled green leaves of *R. madagascariensis* were dried in shed for few days. For solvent extraction 200 g dried leaves of *R. madagascariensis* were put into the thimble of the Soxhlet apparatus and 2 liters of solvent was loaded, following 1:10 ratio, into the solvent chamber. Three different solvents viz. petroleum ether, ethyl acetate and acetone were passed through the column one after another with the same plant material in a non-polar to polar approach. The extraction period was fixed at 72 hours for each solvent with 8 hours maximum a day. Extractives

were collected from the solvent chamber and concentrated through evaporation using a rotary evaporator. The extractives were stored at 4 °C in a refrigerator.

2.4 Rearing of larvae and Mosquito culture

Through standard scooping and dipping method (Robert et al., 2002), larvae of *Culex vishnui* group were collected from flooded rice field of Agriculture Farm, The University of Burdwan (23°16' N, 87°54' E) to set up the colony. The larvae were placed in dirt-free plastic trays filled with tap water and periodically fed with a mixture of brewer yeast, dog biscuits and algae in 3:1:1 ratio (Kamaraj et al., 2011). Pupae were transferred from the trays to insectary (45×45×40 cm) for the purpose of adult emergence. Adults are provided with 10% sucrose solution with multivitamin syrup in a container with a cotton wick. With the help of the keys provided by Barraud (1934), Christophers (1933) and Chandra G (2000) the mosquitoes were identified. On the 5th day of nurturing, a blood meal from a non-motile shaved pigeon was given to adults (females) overnight. To make oviposition possible Petri dishes filled with 100 ml of tap water and wrinkled with filter paper were kept inside the cage. The eggs were undisturbed and allowed to hatch under laboratory conditions. By repeating this process a laboratory reared colony of *Cx. vishnui* group was built up. The colony was kept at a hygienic place and maintained at 27±2 °C temperature and 80–85% relative humidity (RH) under a 13:11 light-and-dark cycles.

2.5 Larvicidal bioassay

According to the standard protocol of World Health Organization (World Health Organization, 2005) the larvicidal bioassay was done at the Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory, The University of Burdwan. Crude extracts were applied against all larval instars of *Culex vishnui* group. Twenty five larvae of different instars (1st, 2nd, 3rd, and 4th) were transferred into sterilized glass Petri dishes of 9 cm diameter with 150 mL capacity. Graded concentrations of crude extracts from 0.1% to 1.0% were applied in each of the Petri dish. Five different working solutions were prepared using each of the solvent extracts ranging from 20 ppm to 100 ppm. Solvent extracts were air dried and applied directly on different Petri dishes containing

100 ml of tap water for carrying out larvicidal bioassays with reference to all the combinatorial larvicidal solutions. Each experiment was done in triplicate (n=75) with a set of control. Petri dishes were kept at room temperature (28 ± 2 °C) and 88±2% relative humidity. 920 mg dried yeast powder was added in each Petri dish as larval food. After 24hrs, 48hrs and 72hrs of post exposures the mortality rates were recorded. The larvae were supposed to be dead when they failed to move after probing with needle in the siphon or cervical region of it or when they could not reach to the water surface (Macedo et al., 1997).

2.6 Phytochemical analyses

The phytochemical analyses of the plant extractives were carried out using the standard protocol of Harborne (1984) and Stahl (1989).

2.7 Effect on non-target organism

Toxicity to non-targets is the major limiting factor in designing newer pesticides. To decide the effect of the bio-active compound on non-target organism, *Chironomus circumdatus* larvae were used. Those larvae were exposed to LC₅₀ value of 3rd instars and mortality rate or others anomalies like listlessness or abridged swimming activity were observed after 72h of post exposure.

2.8 Statistical analyses

Abott's formula (Abott WS, 1925) was used to precise the percentage mortality (%M) throughout the observation. Determination of LC₅₀ and LC₉₀ values of crude and solvent extracts were carried out through Log-probit and regression analyses respectively. ANOVA analyses were done for further statistical justification.

Conflict of interest statement

The authors declare no conflict of interest.

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