J. Entomol. Res. Soc., 13(2): 27-36, 2011 *ISSN:1302-0250*

Relative Larvicidal Efficacy of Three Species of Peppercorns against Dengue Fever Mosquito, *Aedes aegypti* L.

Sarita KUMAR* Radhika WARIKOO Naim WAHAB

Department of Zoology, Acharya Narendra Dev College (University of Delhi), Kalkaji, New Delhi 110019, INDIA
*Corresponding Author, e-mail: sarita.sanjay90@gmail.com

ABSTRACT

The present investigations involve the laboratory study of biocontrol potential of hexane extracts of dried fruits of three species of peppercorns; Long pepper, *Piper longum* L., Black pepper, *P. nigrum,* and White pepper*, P. nigrum* against larval forms of *Aedes aegypti* (Diptera: Culicidae), the vector of dengue haemorrhagic fever. When analyzed individually, hexane extracts of Black *P. nigrum* were found to be most effectual against the early fourth instar larvae of *Ae. aegypti*, followed by *P. longum*, and White *P. nigrum* being least effective. The LC_{50} values obtained with hexane extracts of *P. longum,* White *P. nigrum* and Black *P. nigrum* against early fourth instar larvae were 0.017, 0.024, and 0.007 ppm, respectively and the LC_{90} values were 0.065, 0.081, and 0.027 ppm, respectively. It was observed, however, that the larvae of *Ae. aegypti* were most susceptible against a mixture of the three extracts when taken in 1:1:1 ratio exhibiting the LC_{50} and LC_{90} value of 0.002 and 0.011 ppm, respectively. The larvae treated with all the pepper species showed initial abnormal behaviour in their motion followed by excitation, convulsions and paralysis leading to 100% kills which indicated delayed larval toxicity and effects of the extracts on the neuromuscular system. Observations of morphological alterations on treated larvae under light microscopy revealed that all organs had a normal structural appearance as that of controls except the little structural deformity in the form of shrinkage of internal membrane observed in anal gills. Potency of hexane extracts of dried peppercorns provided an excellent potential for controlling *Ae. aegypti* at the larval stage.

Key Words: Aedes aegypti, *Piper* sp., hexane extracts, bioassay, convulsions, anal gills.

INTRODUCTION

The medical importance of mosquitoes as vectors for the transmission of serious diseases that cause morbidity, mortality, economical loss, and social disruption such as malaria, lymphatic filariasis, and viral diseases is well documented (Becker *et al*., 2003). *Aedes aegypti*, the primary carrier for viruses that cause dengue fever, dengue hemorrhagic fever and yellow fever are widespread over large areas of the tropics and subtropics. At present, no effective vaccine is available for dengue; therefore, the only way of reducing the incidence of this disease is by mosquito control, which is frequently dependent on applications of conventional synthetic insecticides (Malavige *et al*., 2004).

In the past, synthetic organic chemical insecticides-based intervention measures for the control of insect pests and disease vectors have resulted in development of insecticide resistance in some medically important vectors of malaria, filariasis and dengue fever (WHO, 1992). Insecticide resistance is increasingly becoming a problem for many vector control programmes. Resistance may develop due to changes in the mosquitoes' enzyme systems, resulting in more rapid detoxification or sequestration of the insecticide, or due to mutations in the target site preventing the insecticide-target interaction (Hemingway *et al*., 2004). The frequent use of chemical insecticides to manage insect pests leads to a destabilization of the ecosystem and enhanced resistance to insecticides in pests (Kranthi *et al*., 2001) suggesting a clear need for alternatives.

Plants are the chemical factories of nature, producing many chemicals, some of which have medicinal and pesticidal properties (Chansang, 2005). More than 2,000 plants species have been known to produce chemical factors and metabolites of value in pest control programs (Ahmed *et al.,* 1984), and among these plants, products of some 344 species have been reported to have a variety of activity against mosquitoes (Sukumar *et al*., 1991).

During the last decade, various studies on natural plant products against mosquito vectors indicate them as possible alternatives to synthetic chemical insecticides. Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans and other advantages (Liu *et al*., 2000). In addition, increasing documentation of negative environmental and health impact of synthetic insecticides and increasingly stringent environmental regulation of pesticides (Isman, 2000) have resulted in renewed interest in the development and use of botanical insect management products for controlling mosquitoes and other insect pests.

The Piperaceae (pepper) family contains approximately 2,000 species, which are widely grown and commonly used in tropical regions as medicines, spice, and condiments in regional cuisine (Numba, 1993). Pepper plants have also been prescribed for pest control as they contain potentially insecticidal compounds (Su & Horvat, 1981). Some *Piper* spp.; *Piper longum*, *P. nigrum*, *P. guanacastensis*, *P. cubeba* and *P. peepuloides*; and their bioactive constituents are reported to have remarkably larvicidal activity against various mosquito species such as *Culex pipiens pallens*, *Ae. aegypti*, *Ae. togoi,* and *Ae. atropalpus* (Pereda-Miranda *et al*., 1997; Miyakado *et al*., 1989; Lee, 2000, Park *et al*., 2002). Okorie & Lawal (1998) has reported the larvicidal properties of ethanolic extracts of fruits of *P. guineense* (African black pepper) against larvae of *Ae. aegypti* (L). Scott *et al*. (2004) also reported that the extracts from three species of the plant family Piperaceae; *P. nigrum* (L), *P. guineense* and *P. tuberculum* (Jacq.) were effective against insects from five orders. Keeping in view the advantages posed by pepper plants over synthetic insecticides, this study was aimed to evaluate the potential of three pepper plants against the larvae of *Ae. aegypti* through larvicidal bioassays. The behavioural changes and morphological alterations of larvae treated with a lethal dosage of the hexane pepper extracts were also observed.

MATERIALS AND METHODS

Mosquito Culture

The present investigations employ the early fourth instar larvae of *Ae. aegypti* originated from field-collected engorged female adults from Delhi. The colony was maintained in an insectary without any insecticide exposure at 28 $\pm$ 1^{o}C, 80 + 5% RH and 14L: 10D photoperiod (Kumar *et al*., 2002). Adults were supplied with soaked deseeded raisins. Periodic blood meals were provided to female mosquitoes for egg maturation by keeping restrained albino rats in the cages. The eggs were collected in an enamel bowl lined with Whatman filter paper and were allowed to hatch in enamel trays filled with de-chlorinated water. Larvae were fed upon a mixture of yeast powder and grinded dog biscuits. The pupae formed were collected in enamel bowls and transferred to the cloth cages for adult emergence.

Preparation of Extracts

The dried fruits of three *Piper* species of the Piperaceae family, Long pepper, *P. longum* L.; Black pepper *P. nigrum*, and White pepper *P. nigrum*, were commercially obtained. The voucher specimens were preserved for future reference. The 160 g of dried and powdered fruits of peppercorns of each variety was soaked in 250 mL of hexane at room temperature for 5 days. The crude extract, thus formed was separated by suction filtered through a Büchner funnel, and the filtrate was concentrated to dryness with a rotary evaporator at 60°C until the solvent completely evaporated. The extract of each plant was thus obtained, lyophilized, and then refrigerated at -20° C until testing for mosquito larvicidal activity.

Larvicidal Bioassay

The larvicidal bioassay was performed at 28 $\pm$ 1^{o}C on the *Ae. aegypti* larvae in accordance with the WHO method for mosquito larvae (WHO, 1981). The graded series of each extract was prepared using hexane as the solvent. The early fourth instar larvae of *Ae. aegypti*, in batches of 25, were taken in plastic bowls containing 99 mL of distilled water and transferred to glass jar containing 150 mL of distilled water and 1 mL of the particular concentration of hexane pepper extracts. Four replicates were carried out simultaneously for each dilution. Controls were exposed to the solvent, i.e. hexane alone. During the treatment period, the larvae were not provided with any food. The dead and moribund larvae were recorded after 24 hours as larval mortality. The behavioural symptoms of the treated larvae were also recorded at regular time intervals.

Statistical analysis of data

The tests with more than 20% mortality in controls and pupae formed were discarded and repeated again. If the control mortality ranged between 5-20%, it was corrected using Abbott's formula (Abbott, 1925).

$$\text{Corrected Mortality} = \frac{\text{\% Test Mortality - \% Control Mortality} \quad \text{X 100}}{\text{100- \% Control Mortality}}$$

The data were subjected to regression analysis using computerized SPSS 11.5 Programme. The LC_{50} and LC_{90} values with 95% fiducial limits were calculated in each bioassay to measure difference between the test samples. The results obtained with different extracts were analyzed using Student's t-test with statistical significance considered for $P \leq 0.05$.

Morphological Study

After treatment with a lethal dosage (LC_{99}) of each pepper extract, the dead larvae were studied for morphological alterations under light microscopy. Larvae mounted with Hoyer's medium on a microscope slide were scrutinized under light microscopy. Morphological changes in body segments including the head, thorax, and abdomen, and other organs such as the eyes, antennae, setae, and anal gills were observed, and compared with those of the controls.

RESULTS

The results of larvicidal bioassays performed upon the fourth instar larvae of *Ae. aegypti* with the hexane extracts of three pepper species are presented in Table 1. The results obtained ascertain the effectiveness of all the pepper species against the mosquito larvae. All the treatments resulted in complete mortality without any pupal or adult emergence. The control or untreated groups did not show any mortality within 24 h. The larvae developed into pupae and then adults within 48-72 h.

The investigations revealed the LC_{50} and LC_{90} values for the hexane extract of *P. longum,* black *P. nigrum* and white *P. nigrum* as 0.017, 0.007 and 0.024 ppm; and 0.065, 0.027 and 0.081 ppm, respectively (Table 1). These results proved the extracts of black *P. nigrum* being significantly more effective than that of *P. longum* (2.4-fold; $p>0.05$) and of white *P. nigrum* (3.4-fold; $p>0.05$) (Fig. 1).

The most interesting observation was that the hexane extract of the mixture of the dried fruits of *P. longum,* black *P. nigrum* and white *P. nigrum* taken in a 1:1:1 ratio exhibited the highest larvicidal potential against early fourth instar larvae exhibiting an LC_{50} and LC_{90} value of 0.002 and 0.011 ppm, respectively as compared to the three dried peppercorns when tested individually (Table 2). The larvicidal values obtained with mixture extract proved it to possess significantly higher ($p>0.05$) larvicidal potential than the other extracts. The mixture was found 3.5 times more efficient than black *P. nigrum*, 12 times more toxic than white *P. nigrum* and 8.5-fold more mosquiticidal than *P. longum* (Fig. 1).

Table 1. Larvicidal activity of the hexane extract derived from three *Piper* species against fourth instar larvae of *Ae. aegypti.*

***Piper* sp. Extract (ppm)**	**% Mortality (Mean ± S.E.)**	**Larvicidal Activity**		**Slope ± S.E.**	**χ^2(df)**	**Regression coefficient**
		LC_{50}*	**LC_{90}***			
P. longum						
0.006	4 ± 0.58	0.017 b	0.065 d	2.242 ± 0.29	8.60 (6)	2.242
0.008	28 ± 4.60	(0.011 – 0.026)	(0.039 – 0.197)			
0.01	40 ± 11.27					
0.02	60 ± 9.71					
0.04	68 ± 3.56					
0.06	84 ± 12.54					
0.08	100 ± 0.0					
Control	0					
***P. nigrum* (Black)**						
0.002	4 ± 0.36	0.007 a	0.027 c	2.580 ± 0.31	2.79 (8)	2.275
0.004	32 ± 8.90	(0.006 – 0.009)	(0.020 – 0.041)			
0.006	48 ± 10.42					
0.008	52 ± 15.67					
0.01	64 ± 9.85					
0.02	80 ± 17.96					
0.04	96 ± 6.74					
0.06	96 ± 2.87					
0.08	100 ± 0.0					
Control	0					
***P. nigrum* (White)**						
0.006	4 ± 0.58	0.024 b	0.081d	1.501 ± 0.23	9.31 (6)	2.421
0.008	16 ± 4.98	(0.013 – 0.042)	(0.045 – 0.134)			
0.01	28 ± 5.57					
0.02	48 ± 12.45					
0.04	64 ± 17.54					
0.06	72 ± 11.98					
0.08	100 ± 0.0					
Control	0					

* Figures in the column followed by the same letter are not significantly different at p=0.05 (Student's t-test).

Table 2. Larvicidal activity of the mixture of hexane extract derived from three *Piper* species against fourth instar larvae of *Ae. aegypti.*

***Piper* Extract (ppm)**	**% Mortality (Mean ± S.E.)**	**Larvicidal Activity**		**Slope ± S.E.**	**χ^2(df)**	**Regression coefficient**
		LC_{50}	**LC_{90}**			
Mixture of three Extracts						
0.0004	4 ± 0.26	0.002	0.011	1.200 ± 0.14	7.92 (8)	1.830
0.0008	20 ± 6.94	(0.001 – 0.003)	(0.008 – 0.018)			
0.001	44 ± 9.85					
0.002	52 ± 11.27					
0.004	64 ± 8.68					
0.006	72 ± 12.32					
0.008	84 ± 5.45					
0.01	88 ± 0.46					
0.02	100 ± 0.0					
Control	0					

Symptomatological observations on the larvae treated with the three *Piper* extracts and their mixture revealed a similar manner of toxicity in the larvae. All larvae were still active immediately after exposure to LC_{90} of each pepper extract, possessing normal zigzag motion. However, after 5 min of exposure, abnormal evidence of excitation, restiveness, and listlessness was initially observed which persisted for 10-30 min, and was followed by other anomalous motions such as a coiling movement. During the period of 30-60 min, some larvae showed more toxic symptoms including tremor and convulsion at the bottom of the glass jar. Two hours after treatment, more than one-half of the larvae were paralyzed and had sunk to the bottom of the jar. Moribund or dead larvae were increasingly found from 2 to 7 h. At the end of a 24-h exposure period, all larvae had subsequently died.

Observations on the morphological alterations of treated larvae revealed that most organs, except anal papillae (gills), had a usual structural appearance. Under light microscopes, both treated and control larvae showed similarities in morphological structural design and cuticular sculpturing of the head, thorax, and abdomen segments, and other organs such as the eyes, antennae, setae and siphon. A discrete difference, however, was the structural alteration of the anal gills observed in the pepper-treated larvae exhibiting notable shrinkage in the internal structures while the external features were normal in appearance.

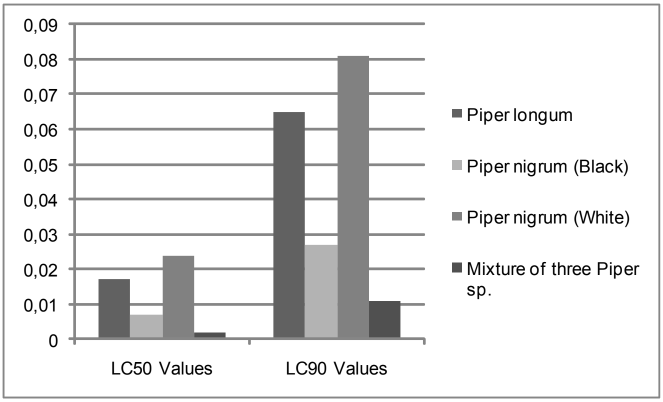

Fig. 1. Larvicidal efficacy of hexane extracts of three peppercorns separately and the mixture of three extracts against *Ae. aegypti*

DISCUSSION

An insecticide does not have to cause high mortality on target organisms in order to be acceptable. The problem of high cost and development of resistance in many vector mosquito species to several of the synthetic insecticides have revived interest in exploring the pest control potentials of plants. Also, economic and environmental concerns have encouraged a tendency recently towards the use of “soft” pesticides. Phytochemicals may serve as the alternate measures being relatively safe, inexpensive and readily available in many parts of the world. Recently, there has been a growing interest in plants belonging to the family Piperaceae as a potential source of bioactive chemical compounds against mosquito vectors (Chaithong *et al*., 2006). So far, at least 611 active ingredients have been isolated and identified from different parts of *Piper* species (Dyer *et al*., 2004). The dried fruits of some Piperaceae are used as flavoring agents in food, but are known to have insecticidal properties (Miyakado *et al*., 1979, 1989; Su & Horvat, 1981).

The objective of the present study was to evaluate the larvicidal potential of three different species of dried peppercorns against the early fourth instar of *Ae. aegypti.* Quantification of vector behavior, other than toxicity, in response to chemical exposure is useful in understanding the mode of action of irritants and/or repellents on target physiological sites (Licciardi *et al*., 2006). Such information is important in generating more effective and safer chemicals for public health purposes, and can aid in the development of innovative vector control strategies.

Our studies confirmed the larvicidal potential of three species of hexane pepper extracts against the Indian strain of *Ae. aegypti*. Against early fourth instars the hexane extracts of a mixture of *P. longum,* Black *P. nigrum* and White *P. nigrum* proved to be the most toxic; being 3.5-12 times more efficient than the individual extracts. Whereas the extracts when analyzed separately revealed White *P. nigrum* to be the least effective amongst the three pepper species. It was found that the extracts of Black *P. nigrum* were 2.4 times more effective than that of *P. longum* and 3.5 times more efficient than that of white *P. nigrum.* In 2007, Oke *et al*. had reported the larvicidal potential of the hexane extract of *P. guineense* due to its high toxicity, environmental compatibility and non-persistence. In a Thailand strain of *Ae. aegypti*, Chaithong *et al.* (2006) reported that ethanolic extracts of *P. longum* showed more remarkable larvicidal potential than *P. sarmentosum* and *P. ribesoides*, presenting LC_{50} and LC_{95} values of 2.23 and 4.80, 4.06 and 12.06, and 8.13 and 14.01 ppm, respectively. Earlier, Chansang *et al.* (2005) recorded 79 mg/L and 229 mg/L as the LC_{50} and LC_{90} values when the early fourth instar larvae of a Thailand strain of *Ae. aegypti* were treated with aqueous extract of *P. retrofactum*. In an Indian strain of *Cx. quinquefasciatus*, Vasudevan *et al.* (2009) obtained 29.11 and 62.37 mg/L, as the LC_{50} and LC_{90} values, respectively when the early IV larval instars were treated with ethanolic extracts of *P. nigrum.* According to them as the dried fruits of *P. nigrum* are available most of the time throughout the country, the larvicidal properties of the plant species can be well-utilized, while planning alternate vector control programmes. A piperidine alkaloid, pipernonaline, was found to be responsible for this activity, with the 24-h median lethal dose (LD_{50}) value for this compound being 0.21 mg/L. Amer and Mehlhorn (2006) also reported the larvicidal effects of the black pepper oil against the third instar larvae of *Ae. aegypti*, though according to their investigations efficacy of black pepper oil depends upon the storage conditions as only one week of storage of oil under different conditions led to loss of its toxicity.

The biological activity of the ethanolic extracts of *P. nigrum* might be due to the various compounds including potentially insecticidal compounds (Su & Hovrat, 1981). The studies have shown that the component, pipernonaline, isolated from the hexane fraction of the *P. longum* fruit possessed potent larvicidal activity against *Ae. aegypti* with LC_{50} of 0.25 mg/L. (Lee, 2000; Yang *et al*., 2002). Unsaturated amides constitute the major group among the natural products identified in these fruits. Several insecticidal amides, such as pipericide, (E,E)-N-(2-methylpropyl)-2,4,12-tridecadienamide, and (E,E,E)-11-(1,3-benzodioxol-5-yl)-N-(2-methylpropyl)- 2,4,10-undecatrien-amide, have been isolated from *P. nigrum* (Miyakado *et al*., 1979; Su & Horvat, 1981). Neurotoxic amides and lignans appear to be mainly responsible for the anti insect activities of *Piper* species (Greger, 1988; Gbewonyo *et al*., 1993). The promising *Piper*-derived insecticides are results of the search for new phytochemical agents from Piperaceae plants, which should influence further research of other plants belonging to this family in order to find affordable natural substances for use in mosquito control.

The gradual anomalous behaviour in the motion of treated larvae resulting in 100% kill indicates a delayed type of larval killing from the pepper extracts. Our studies also showed the alterations in the anal papillae with considerable shrinkage of the internal membrane. These investigations are in accordance with the results of Chaithong *et al*. (2006) who also reported the remarkable shrinkage in the internal structure of anal papillae in the larvae of a Thailand strain of *Ae. aegypti* when treated with ethanolic extracts of black pepper, while most of the other organs of dead larvae had a normal appearance. Likewise, *Cx. quinquefasciatus* larvae treated with ethanolic extract of *Kaempferia galanga* revealed the severely morphological disruption of anal papillae with a shrunken cuticle border and destroyed surface with loss of ridge-like reticulum under light and scanning electron microscopy, respectively (Insun *et al*., 1999). Green *et al*. (1991) also reported swollen anal papillae in *Ae. aegypti* larvae after treatment with whole oil of *Tagetes minuta*. Chaithong *et al.* (2006) suggested that structural deformation of anal papillae probably led to their dysfunction, which may be intrinsically associated with the death of mosquito larvae. Earlier, it was reported that uptake and elimination of most ions in mosquito larvae occur via the anal papillae, which was markedly reduced or lost in papilla-less larvae (Garrett & Bradley, 1984; Clements, 1992). This indicated that the lack or dysfunction of the anal papillae probably led to an interruption of the osmotic and ionic regulation.

Variety of types and levels of active constituents in each *Piper* species may be responsible for the variability in their potential against *Ae. aegypti*. Our investigations demonstrated and emphasized the potential of Piperaceae plants against *Ae. aegypti* larvae and its benefit to developing new types of larvicides used for mosquito control. However, further research needs to be carried out in order to determine the mode of action and elucidate the active ingredients present in the plant.

ACKNOWLEDGEMENTS

The authors are grateful to University Grants Commission for providing the financial assistance to our project. The authors extend thanks to Dr. Savithri Singh, Principal, Acharya Narendra Dev College for providing the laboratory and culture facilities to conduct the experimental work.

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Received: June 06, 2010   Accepted: March 08, 2011