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**Research Article**

# Effect of *Citrus hystrix* DC Leaves Ethanol Extract on Larvae of *Aedes aegypti*

**M. M. Mya, Y. Y. Aye, A. W. Oo and R.K. Saxena***

*Medical Entomology Research Division, Department of Medical Research (Lower Myanmar), Dagon Township, Yangon, Myanmar*

**Centre for Biomedical Engineering, Shobhit University, Modipuram, Meerut-260110, India*

**Email: rksaxena369@gmail.com*

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**Abstract:** Vector Control Program is an important public health mission for the eradication of vector-borne disease. *Aedes aegypti* mosquito is a main vector for transmitting the viruses of dengue. Eradication of *Aedes aegypti* at larval habitat from the domestic environment is the only way to prevent dengue transmission. In present study *Citrus hystrix* (Kaffir lime) leaves ethanol extract was used for the elimination of larvae of *Aedes aegypti*. Larvicidal effects of Citrus extract in different dilutions on larvae of *Aedes aegypti* were monitored according to WHO Guidelines for Laboratory Testing of Mosquito. Results of study found that at 2.4%, 2.1%, 1.8%, 1.5% and 1.2% concentrations of *Citrus hystrix* leaves ethanol extract caused 99.5%, 85.5%, 62.5%, 26.5% and 2% mortality of Aedes larvae in 24 hrs. respectively. However 1.2% concentration showed almost negligible larvicidal effect. Result of study suggests that high concentrations of *Citrus hystrix* leaves ethanol extract can be used for the eradication of *Aedes aegypti*.

## INTRODUCTION

The mosquitoes are potential vectors of many diseases, including malaria, filariasis, dengue, cerebral encephalitis and yellow fever etc. Mosquito's vector borne diseases are causing huge human suffering. In Myanmar mosquito borne diseases are causing huge loss of life in rural and urban areas and large portion of health fund goes for the treatment and control programs, however mosquito diseases continue to explode from time to time and major public health problem in Myanmar [1]. There is an urgent need to check the proliferation of the population of vector mosquitoes in order to reduce vector borne diseases by appropriate control methods. Dengue vector mosquitoes and other species of mosquitoes developed insecticides and chemicals resistance and causing major concern for the global effort to control mosquito borne diseases as Dengue fever (DF), Dengue haemorrhagic fever (DHF) Yellow fever and malaria [2, 3]. Hence, elimination of mosquitoes and the diseases spread of outbreak by them are a continuous process. Eradication of mosquito's vector borne diseases can be controlled by elimination of mosquitoes to protect mankind. Several mosquito control/eradications methodologies have been developed depending upon the condition of vector larvae and adults by synthetic chemical pesticides, biological-control agents and trapping [4]. Also researches are going on around the world genetically modified neutral mosquitoes [5]. Synthetic insecticide-based interventions for mosquito-borne diseases leads to multifarious problems i.e. it disperse through the air, soil and water and create insecticide resistance mosquitoes and generate toxic hazards to human and animals and environmental pollution [6-8]. Adverse effects of available insecticides on life system desire development of environmental friendly substitute for mosquito eradication program. Cheng et al. 2009 suggested the application of natural materials as a larvicidal agent for decreasing the harmful effects of chemical pesticides on environment [9]. Literature survey suggests that plants and microorganisms can provide alternatives to chemical insecticides, because they are safe and easily available and will not produce any pollution [10]. Several studies showed that plant extracts or essential oils can provide desired larvicidal properties against different species of mosquitoes without causing bad effects to humans [11-16]. So mosquito larvicidal agents from plants and microorganisms can be used as effectively without causing any ill effects to living being. Also plant insecticides can be easily procured due to its natural source supply; they are economical and are eco-friendly. Bio based insecticides are generally nontoxic to human and environment [17, 18]. Studies found that *Citrus* family plant has enormous potential for controlling the population of larvae and adults of different types of mosquitoes [19-21]. *Citrus hystrix* DC. (Kaffir lime) is a member of *Citrus* family and its leaves, fruits, barks and roots are being used in tropical Asia for culinary and traditional medicine purposes. *Citrus hystrix* plants are also available in many parts of the Myanmar and plants are abundantly present in coastal areas of Thaninthayi Region and Mon State. Studies observed that bioactive components isolated from the

alcoholic and hexane crude extracts of *Citrus hystrix* plant possess several insecticide and protective properties i.e. Antibacterial, antifungal and mosquitocidal effects against *Aedes aegypti* fourth instar larvae [22-24]. The present study is done to investigate the effect of ethanol leaves extract of *Citrus hystrix* for larvicidal properties of *Aedes aegypti*. The present study may provide beneficial information on the bioactive component obtained from native plant source and can help in the development of new mosquito control agent by using the WHO prescribed methodology.

## MATERIALS AND METHODS

### Collection, extraction and preparation of ethanol *Citrus hystrix* DC leaves extract

*Citrus hystrix* DC (Kaffir lime) is a thorny bushy plant found in tropical Asian countries. In Myanmar it is found abundantly in coastal areas of Thaninthayi Region and Mon State. The mature plant is about 1.8 to 10.7 m tall with aromatic and distinctively shaped "double" leaves. The fruit of *citrus* lime is a rough, bumpy green fruit. The leaves of *Citrus hystrix* plant is dark green color with a glossy sheen, and is composed of two leaveslets. The top leaveslet is light in shade.

### Collection of Leaves

3 kilograms of healthy and fresh leaves of *Citrus hystrix* DC were obtained from local farm of Mawlamyine Township, Mon State. Leaves were thoroughly washed 2–3 times with distilled water and shade dried for 30 days at room temperature. The extraction procedure was done at Central Research and Development Center (CRDC) Myanmar.

### Extraction procedure

After shade drying for 30 days 100 grams of dried were ground to make powder and were dissolved in 500 ml 95% ethanol 1:5 wt./v. in1000 ml capacity round conical flasks by cold percolation method at ambient temperature of 30°+C in Biochemistry Laboratory for 7 days. The heads of conical flask was sealed thoroughly by 0.016 mm thick aluminum foil for the prevention of vaporization and contaminations. Ethanol and dries leaves powder in conical flask mixed thoroughly by hand so all contents should be distributed uniformly. After 7 days leaves ethanol mixture was then filtered through Whattman filter paper 500 ml in Erlenmeyer Pyrex conical flask, to remove the undissolved plant materials. Ethanol solvent from leave extract in Pyrex conical flask was removed by vacuum glass rotary evaporator to yield concentrated leave extract. It is found that 100 gm. of dried *Citrus* leaves produced 8 gm. of viscous extract of *Citrus hystrix* leaves. Viscous extract was stored at 4°C in Refrigerator until use for their evaluation as *Aedes aegypti* mosquito larvicidal agent.
In earlier preliminary study we observed larvicidal effect by leaves extract of *Citrus hystrix* DC on larvae of *Aedes aegypti* (Unpublished data). In present study we have made dilutions of leaves extract of *Citrus hystrix* DC with distilled water of 5 gm. extract i.e. 2.4%, 2.1%, 1.8%, 1.5% and 1.2% concentration in 100 ml distilled water in 150 ml plastic cups.

### Procurement of larvae for testing the effect of *Citrus hystrix* DC

Dagon Township strain of *Aedes aegypti* larvae reared in laboratory were used and 50 each third and fourth instars larvae were collected and kept in five plastic cups (150 ml) containing 2.4%, 2.1%, 1.8%, 1.5% and 1.2% concentration of leaves ethanol extract of *Citrus hystrix* in 100 ml distilled water and one plastic cup with 100 ml distilled water only as control. In each tray 300(50 larvae x 6cups) larvae of *Aedes aegypti* was put to study the effect of leaves ethanol extract of *Citrus hystrix.*

### Larvicidal testing procedure

Before each experiment fresh dilutions were prepared of ethanol extract of *Citrus hystrix* leave in distilled water. Fresh different concentration of leaves ethanol extract of *Citrus hystrix* DC i.e. 2.4%, 2.1%, 1.8%, 1.5% and 1.2% were prepared in 100 ml in distilled water in 150 ml plastic cups. Fifty (50) each 3rd and 4th instars *Aedes aegypti* larvae were put into different concentrations of *Citrus hystrix* also a blank control having only distilled water test was done simultaneously. Detail testing was done according to standard method (WHO 1963). Larvae were exposed for 24 hrs. in each concentration solution in laboratory at 27-29°C ambient temperature and 70 to 80% relative humidity. Four replicate of larvicidal study were done and mortality of *Aedes aegypti* larvae were checked and recorded after 24 hrs. of exposure period. Dead larvae were identified when the larvae failed to move after probing with a needle in the thorax region of the body. Data of larvicidal effect of *Citrus* ethanol extract were recorded and analyzed by Microsoft Excel software. The lethal concentrations of $LC_{50}$ and $LC_{90}$ values for 95% confident limits were calculated after 24 hrs. using dose-effect probit analysis graph [25,26].

$$X^2 = (O-E)^2/Ex(100-E)$$

where: $X^2$=Chi squire;
O=Observed value;
E=Expected value;
O-E= Observed minus expected

## RESULTS AND DISCUSSION

Effect of ethanol extract of *Citrus hystrix* leaves in different dilutions on the larvae of *Aedes aegypti* was studied to evaluate larvicidal action, because bite of *Aedes* can lead to several fatal ailments. Also, attempt was made to develop an environment friendly method for the elimination of *Aedes aegypti*. Larvicidal of *Aedes* mosquitoes were measured at Entomology Laboratory, Yangon, Myanmar. Third and fourth instars age groups *Aedes aegypti* larvae reared at ambient temperature of 27-29°C with relative humidity of 70-80% and served as a test organism material. Results with different dilutions of *Citrus hystrix* leaves ethanol extract demonstrated significant mortality of *Aedes aegypti* larvae (Table 1).

**Table 1. Effect of ethanol extract of *Citrus hystrix* DC leaves on 3rd and 4th instars *Aedes aegypti* larvae**

| Ethanol extracts centration (%) | Mortality | | | | | Total tested larvae | Mortality | % Mortality |
|---|---|---|---|---|---|---|---|---|
| | Test larvae | Test 1 | Test 2 | Test 3 | Test 4 | | | |
| 2.4 | 50x4 | 50 | 49 | 50 | 50 | 200 | 199 | 99.5 |
| 2.1 | 50x4 | 44 | 45 | 44 | 46 | 200 | 179 | 85.5 |
| 1.8 | 50x4 | 32 | 34 | 31 | 28 | 200 | 125 | 62.5 |
| 1.5 | 50x4 | 15 | 12 | 12 | 14 | 200 | 53 | 26.5 |
| 1.2 | 50x4 | 0 | 1 | 3 | 0 | 200 | 4 | 2 |
| Control | 50x4 | 0 | 0 | 0 | 0 | 200 | 0 | 0 |

This indicates that *Citrus hystrix* leaves ethanol extract has an active component which produced high casualty to *Aedes aegypti* larvae and may be used as important agent in Dengue eradication program. Results of Table (1) also show that the highest concentrations of ethanol extract of *Citrus hystrix* leaves 2.4% produced 99.5 % mortality of larvae of *Aedes aegypti*, followed by 2.1% *Citrus* leaves extract caused 85.5% mortality of 3rd and 4th instars *Aedes* larvae. However 1.8% concentration of leaves ethanol extract was found to produce only 62.5% mortality of *Aedes* larvae after 24 hours. Then, data of the dead larvae were analyzed by probit and Microsoft Excel software after 24 hrs using dose-effect. In Table (2) the effectiveness of lethal concentration of *Citrus* leaves extract on 3rd and 4th star larvae of *Aedes aegypti* at $LC_{50}$ at 95% confidence corrected limit for 50% mortality and in similar manner in $LC_{90}$ at 95% confidence corrected limit for 90% mortality were analyzed. The highest larval mortality was observed in the third and fourth instar larvae at values of $LC_{50}$ and $LC_{90}$ and it was found to be 1.73% and 2.08% from *Citrus hystrix* leaves ethanol extract. It is found that confidence limit for $LC_{50}$ ranged between was 1.77-1.69% and in $LC_{90}$ confidence limit ranged between 2.14-2.03% in ethanol leaves extract of *Citrus hystrix*.

**Table 2. $LC_{50}$ and $LC_{90}$ of ethanol extract of *Citrus hystrix* DC leaves on 3rd and 4th instars *Aedes aegypti* larvae**

| Treatment | Hours | Slope | $X^2$ | df | $LC_{50}$ corrected limit | $LC_{90}$ corrected limit | 95 % confidence interval (upper and lower limit) |
|---|---|---|---|---|---|---|---|
| *Citrus hystrix* DC leaves ethanol extract | 24 | $LC_{50}$ 1.183<br>$LC_{90}$ 1.208 | 0.445 | 4 | 1.73% | 2.08% | 1.77 to 1.69%<br>2.14 to 2.03% |

## Discussion

In tropical countries of Asia dengue is a serious health problem and causes large number of fatalities. It is caused by dengue virus and the disease is transmitted through *Aedes aegypti* mosquitoes. It has been observed that the prevention of dengue can be efficiently controlled by controlling the vector mosquito at larval stage and it can check the spread of mosquitoes and dengue [27]. Several synthetic and non-synthetic chemical are used for controlling *Aedes aegypti* larvae, but recent studies observed that *Aedes aegypti* larvae are getting resistant to present days chemical compounds [28-30]. Clinical studies found that exposure to synthetic chemical compounds used for controlling dengue mosquito's causes several side effects in human being and may lead to genesis of cancer and hazardous effects in environment [31, 32]. Hence, there is need to develop an ecofriendly natural insecticide for eradication of vector *Aedes aegypti* for controlling dengue in human being.

In present study, an attempt has been made to develop environment friendly chemical compound from herbal plant for the elimination of dengue transmitting vector *Aedes aegypti*. It is found that natural plant compound can perform perfect effect on the elimination of *Aedes* mosquitoes without causing any ill effect on human and animals [33]. In our study thorny bush plant *Citrus hystrix* DC which is native of tropical Asia and commonly used for cooking, traditional medicine and perfumery, was selected and used as an insecticidal agent for the larvae of *Aedes aegypti*. Earlier chemical studies observed that Leaves extracts of *Citrus hystrix* contains flavonoid, saponin, steroid, and terpenoid metabolites [34-36]. Results of study found that 2.4% concentration of leaves extract of *Citrus hystrix* produced maximum 99.5 % mortality of *Aedes* larvae and 2.1% leaves extract caused 85.5% mortality of 3rd and 4th instars *Aedes* larvae. But, 1.8% concentration of ethanol leaves extract caused only 62.5% mortality of *Aedes* larvae within 24 hours (Fig.).

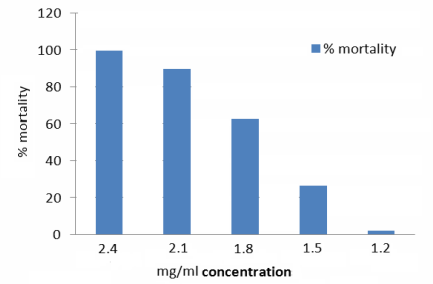


**Figure: Percentage mortality of 3rd and 4th instars *Aedes aegypti* larvae with different dilution of leaves extract of *Citrus hystrix* DC.**

Probit analysis of *Aedes aegypti* mosquito larvicidal effect with different concentrations of *Citrus hystrix* (Finney Statistical regression method used for sigmoid dose-response curve between binomial response variables) and evaluation of significance of the difference between two means were done by Student's t test showed that the effective lethal concentration at $LC_{50}$ for 50% mortality and $LC_{90}$ for 90% produced 1.73% and 2.08% mortality with ethanol leaves extract of *Citrus hystrix*. Fan et al., 2011 observed that leaves oil of *Citrus hystrix* 29.254 and 26.748 ul/g in caused 50% mortality at 24 hours and 48 hours after treatment respectively and Beta-citronellal 66.65% was the most abundant compound in the leaves oil of *Citrus hystrix*. Several researchers have used safer and human and environment friendly dengue controlling methodologies. For elimination of transmitting vector *Aedes aegypti* and thus led to a situation where researches regarding biolarvicidal agents using plants as its main component [35-40]. However, Buatone and Indrapichate showed that 6.4% ethanol extract of *Citrus hystrix* leaves caused 90% mortality adult rice weevils; however water leaves extracts produced low mortality effect than the ethanol leaves extract [41]. But Nataya et al., (2010) found that *Citrus hystrix* (3.36%) contains more oil content than *S. aromaticum* (1.50%) in dry weight and *Citrus hystrix* caused high larvicidal effect on both pyrethroid susceptible

and resistant strains of *Aedes aegypti* with dose dependent [42]. The present study observed that $LC_{50}$ and $LC_{90}$ values were found at 95 % confidence level (upper and lower limit) 1.73% and 2.08% and were very effective larvicidal effect against *Aedes* larvae. Our results are similar to earlier lervicidal studies. Our findings show that the leaves extract of *Citrus hystrix* can be effectively used for larvicidal activities against *Aedes* mosquito larvae. *Aedes aegypti* plays in important role in dengue fever and dengue heamorrhagic fever transmission, and local plant *Citrus hystrix* leaves extract can be used for the control of *Aedes* mosquito population. Also, use of nature plant products can be considered as one of the ecofriendly policy for elimination of pest/insect for eradication of mosquito larvae.

## CONCLUSIONS

**C**hemical insecticides, which are commonly used for the controlling mosquitoes, are not found safe for human being. Hence, there is a need to develop a safe and economical biocompatible insecticide for the prevention of vector mosquitoes. *Aedes aegypti* mosquito is a vector for transmitting several fatal diseases in human beings such as Dengue, Chikungunya and Yellow fever. *Aedes* mosquitoes are commonly found in tropical and subtropical countries. In recent years it is observed that plant extracts and oils can provide a potential alternative to chemical insecticides for the prevention of mosquito vectors. Plants insecticides are generally safe and compatible with human life and environment. *Citrus hystrix* DC (Kaffir lime) plants are commonly found in South East Asia. In present study *Citrus hystrix* ethanol leaves extract was used to assess its efficacy for the control of *Aedes aegypti* at larval stage in domestic environment. 2.4%, 2.1%, 1.8%, 1.5% and 1.2% dilutions of ethanol *Citrus* leaves extract in distilled water were prepared. Larvae of *Aedes aegypti* were immersed in above mentioned different dilutions of *Citrus hystrix* for studying larvicidal effect. In each dilutions 50 x 4 raplicates =200 larvae of *Aedes* mosquito of third and fourth instars stage were placed for 24 hours at ambient environment. *Aedes* mosquito larvicidal effects by different dilutions of *Citrus* leaves extract were monitored after 24 hours. WHO Guidelines were used for monitoring larvicidal effect of *Citrus* on *Aedes aegypti*. Study found that 2.4 and 2.1 % concentrations of *Citrus hystrix* DC in distilled water produced 99.5% and 85.5%mortality of larvae of *Aedes aegypti,* whereas 1.8%, 1.5% dilutions caused 62.5%, 26.5% mortality in ethanol extract of *Citrus hystrix* in distilled water against *Aedes* larvae in 24 hrs. and 1.2% concentration showed negligible larvicidal effect. Results of present investigation indicate that high concentrations of ethanol extract of *Citrus hystrix* dilutions can provide an effective insecticidal agent for the prevention of *Aedes aegypti* mosquito vector. Also, study suggests that plants extracts etc. can be used for the control mosquitoes without causing ill effects on human being and environment.


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**COMPETING INTERESTS:** The authors have declared that no competing interests exist.


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## About Authors



**Dr. Maung Maung Mya** is serving as a Research Officer in the Department of Medical Research, Yangon Myanmar. He did BSc (Hon.) & MSc (Parasitology) Yangon University. He has been awarded Ph.D in Biomedical Engineering at Indian Institute of Technology, Delhi in 2003. He has participated in WHO Workshop, Delhi (1997) & WHO Workshop in Myanmar (2011) on Malaria. In 2014, participated in Malaria Forum Meeting at Kunming Medical University,China. His area of interest are Parasitology, Immunology, Therapeutics, Diabetes mellitus, Thalassemia and Osteoarthritis etc. Received Young Scientist

Award in 1998, Dorga Award 2003, FITT Award 2003, Jawaharlal Nehru Memorial Prize 2005, Good Public Staff Award 2007 and Golden Jubilee Award for Research in 2013. His research works has been appreciated in Myanmar Health Research Conferences.



**Prof. R.K Saxena** obtained Master Degree from All India Institute of Medical Sciences in 1970 and Ph.D. in Biomedical Engineering from Indian Institute of Technology Delhi in 1990, He has produced more than 18 Ph.D. scholars and guided several M. Tech. projects during his tenure. As a teacher, he has taught MBBS, B Tech, M Tech & Ph.D. and Physiotherapy students during span of 40 years. He has published more than 120 research papers in prestigious International & National journals and presented more than 250 papers in Conferences, Work Shop and Seminars in India and abroad. He has received several prestigious awards: two times Jawaharlal Research Prize (1997 & 2005) and 2 times Institution of Engineers Award for Research papers. His field of interests is Experimental Biomechanics, Rehabilitation, and Orthopedics BME.

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