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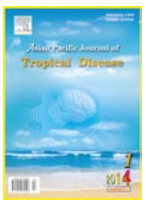

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Mosquito larvicidal efficay of *Acorus calamus* extracts against *Aedes aegypti* L. larvae

Hashmat Imam[1*], Zarnigar[1], Ghulamuddin Sofi[2]

[1]*Department of Preventive and Social Medicine, National Institute of Unani Medicine, Bangalore, India*

[2]*Department of Ilmul Advia, National Institute of Unani Medicine, Bangalore, India*

PEER REVIEW

Peer reviewer

Dr. Nasim Ahmad Khan, Lecturer, Department of Physiology, HSZS Government Unani College, Bhopal 462001 (M.P.) India.
Tel: +91 755 2805801
E-mail: nasimalig@yahoo.com

Comments

The authors have evaluated the mosquito larvicidal activity of *A. calamus* for its biomedical application. Based on the results the authors have proposed that crude extracts of *A. calamus* showed good larvicidal activity. In general the article is well organized; materials and methods appear to be reproducible. The results of the present studies are noteworthy and there is a high possibility of developing an ecofriendly phyto-insecticide.
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ABSTRACT

Objective: To evaluate the larvicidal activity of petroleum ether and ethyl alcohol extracts of *Acorus calamus* (*A. calamus*).
Methods: Petroleum ether and ethyl alcohol extracts were extracted from plant materials through soxhlet extraction process and its efficacy was determined through bioassay method. Extracts were evaluated further for the determination of their LC_{50} and LC_{90} values. Observation of mortality response was assessed after 24 h.
Results: Petroleum ether and ethyl alcohol extracts of *A. calamus* produced 99% and 96% mortality at 125 mg/L respectively. Petroleum ether extract exhibited LC_{50} at 57.32 mg/L, LC_{90} at 120.13 mg/L, while ethyl alcohol extract exhibited LC_{50} at 64.22 mg/L, LC_{90} at 130.37 mg/L.
Conclusions: Present study indicated that *A. calamus* carries huge potential as a mosquito larvicide. This potential could be exploited for the development of safer and effective botanical mosquito larvicidal tool for the management of *Aedes aegypti*.

KEYWORDS
Mosquito, Larvicide, *Acorus calamus*, Extract, *Aedes aegypti*, LC_{50}, LC_{90}

1. Introduction

Mosquitoes belong to the family Culicidae within the order Diptera[1]. There are approximately 3400 species and 42 genera in the world[2]. It transmits a number of diseases, such as malaria, filariasis, dengue, Japanese encephalitis, yellow fever *etc.*, causing millions of deaths every year[3]. *Aedes aegypti* (*Ae. aegypti*) is a vector of dengue and yellow fever while these diseases are widely distributed and continue to be a major public health problem in most tropical and sub tropical areas. Today, about two fifth of the world's population is at risk for dengue, with cases reported in more than 100 countries. In 2007 alone, there were more than 890000 reported cases of dengue in the Americas, of which 26000 cases were of dengue hemorrhagic fever[4,5]. An estimated 200000 persons suffered from yellow fever world-wide each year and the disease causes an estimated 30000 deaths[6]. However, control of dengue and other mosquito-borne diseases is becoming increasingly difficult because the effectiveness of vector control has declined due to the development of resistance in mosquitoes against currently used insecticides[7,8]. Therefore, an effort is made to find alternative insecticides. This has necessitated the continued effort for the search and development of environmentally safe, biodegradable and low cost larvicides

*Corresponding author: Hashmat Imam, Department of Preventive and Social Medicine, National Institute of Unani Medicine, Kottigepalya, Magadi Main Road, Bangalore, India.
Tel: +91 8095142626
E-mail: mdhashmatimam@gmail.com

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for killing larva of mosquitoes from natural sources[9]. Plant extracts are safer for non target organisms including man. Therefore, plant based formulations would be more feasible environmental products with proven potential as insecticide or repellent. It can play an important role in the interruption of the transmission of mosquito-borne diseases at the individual as well as at the community level[10]. Therefore, in response to the urgent need for new, affordable, effective and environment-friendly mosquito control agents; we screened a medicinal plant *Acorus calamus* (*A. calamus*) of the family Acoraceae for mosquito larvicidal activity against *Ae. aegypti*. This plant is commonly distributed throughout the tropics and subtropics, especially in India and Sri Lanka. It is found in marshes, wild or cultivated, ascending the Himalayas up to 1 800 m in Sikkim[11]. It is one of the most valuable plants in the medical sciences almost throughout the India. In Ayurvedic and Unani classical text book its insecticidal and larvicidal activity against many insects has been reported[12]. The rhizome is widely used in a number of ailments like mental ailments, epilepsy, memory enhancing, stimulant, chronic diarrhoea, dysentery, bronchial catarrh, kidney and liver troubles, rheumatism, sinusitis and eczema[13-15].

2. Materials and methods

Study was conducted after obtaining the ethical clearance by the Institutional Animal Ethics Committee (IAEC) of National Institute of Unani Medicine, Bangalore, India under Reg. No-IAEC/VII/04/TST.

2.1. Plant materials

Fresh rhizomes of *A. calamus* was procured from Foundation for Revitalisation of Local Health Traditions, Bangalore, identified by Botanist Dr Ravi Kumar at Foundation for Revitalisation of Local Health Traditions, Bangalore, and voucher specimen has been deposited in the herbarium at National Institute of Unani Medicine, Bangalore, India.

2.2. Preparation of extracts

The rhizomes of *A. calamus* was carefully washed and rinsed with tap water for at least 30 min. Dead rhizomes were removed. Roots were separated from the rhizomes, and shade dried at room temperature (28±1) °C for 15 d. Dried rhizomes were pulverized in electric grinder in the form of coarse powder at pharmacy of National Institute of Unani Medicine, Bangalore. A total of 250 g coarse powder was extracted in Soxhlet extractor with 1 000 mL petroleum ether (Sigma Aldrich, Bangalore) and then with ethyl alcohol (99.99% analytical grade, Changshu Yangguan Chemical, China) at the temperature of 50 °C till discolouration. The liquid extract of each type were cooled and filtered by Whatman filter paper 40. The filtrates were evaporated under reduced pressure at 45 °C to dryness with the help of rotary vacuum evaporator (Heidalph HB Digital). The resultant brownish black crude petroleum and ethyl alcohol extracts were kept in Petri dish and stored in vacuum desiccators.

2.3. Rearing of larvae

The *Ae. aegypti* larvae were reared at National Institute of Malaria Research, Bangalore, an egg strip of F12 generation was obtained from a maintained colony. Eggs strip was dipped into a plastic tray (20 cm×15 cm×5 cm) containing dechlorinated tap water for hatching. To reduce variation in adult size at emergence, larvae were reared at a fixed density of 800-1 000 larvae per tray. Larvae were fed once a day initially and twice during the later stages of development with a diet of finely ground brewer yeast and dog biscuits (3:1)[16]. Adults were fed with 10% sucrose solution. Five days after emergence, female mosquitoes were allowed to blood-feed on albino mice for 2-3 h. A few days after having a blood meal, the gravid mosquitoes laid their eggs. Small plastic bowl having 250 mL of tap water lined with filter paper was kept inside the cage for oviposition. The laboratory colony was maintained at 25-30 °C and 80-97% relative humidity under a photoperiod of 14:10 hours light and dark as per the procedure of Sharma and Saxena (1994). Under these conditions the full development from egg to adult lasted about three weeks[17,18].

2.4. Preparation of stock solutions and test concentrations

Dried extracts of *A. calamus* were dissolved separately in dimethyl sulphoxide (Sigma Aldrich, Bangalore) to prepare dilute solutions. Homogeneous suspensions were obtained by gentle shaking or stirring. A volume of 20 mL 1% stock solution was obtained by weighing 200 mg of the technical material and adding 20 mL solvent to it. It was kept in a screw-cap vial, with aluminium foil over the mouth of the vial. The mixture was shaked vigorously to dissolve the material in the solvent. Test concentrations ranging from 25 to 125 mg/L were obtained by adding appropriate dilution to 250 mL chlorine free or distilled water. The plain control solution was made with 1 mL of dimethyl sulphoxide with 249 mL of dechlorinated water. For other volumes of test water, aliquots of dilutions added were adjusted. While making a series of concentrations, the lowest concentration was prepared first. Small volumes of dilutions were transferred to test beakers by pipettes with disposable tips.

2.5. Larvicidal testing

Bioassay was performed according to WHO guidelines[19]. After making test concentration, 3rd and 4th instar larvae were introduced into each plastic bowel (500 mL capacity). Small, unhealthy or damaged larvae were removed. Each experiment was performed in four replicates with a final total of 100 larvae for each concentration. Each batch of replicates contained one plain control. The number of dead larvae at the end of 24 h was recorded in the data record form. During the treatment no food was offered to larvae. Moribund larvae were counted

and added to dead larvae for calculating mortality percentage. Initially the mosquito larvae were exposed to a wide range of test concentrations. After determining the mortality of larvae in this wide range of concentrations a narrow range of 4–5 concentrations yielding between 10% and 99% mortality in 24 h were used to determine lethal concentration that killed 50% and 90% larval population (LC_{50} and LC_{90}).

2.6. Statistical analysis

Data from all replicates were pooled for analysis. LC_{50} and LC_{90} values were calculated using SPSS software (IBM SPSS Statistics v20–64bit) by probit analysis. The 95% confidence intervals values, and degrees of freedom (*df*), *Chi*-square (χ^2) goodness of fit test and regression equations were recorded. Whenever the χ^2 was found to be significant ($P<0.05$), a heterogeneity correction factor was used in the calculation of confidence limits. The mortality in control group if between 5% and 20% necessitated that the mortalities of treated groups to be corrected according to Abott's formula[20].

$$\%\ \text{Corrected mortality}=\frac{\%\ \text{killed in treated}-\%\ \text{killed in control}}{100-\%\ \text{killed in control}}\times 100$$

3. Result

The efficacy of petroleum ether and ethyl alcohol extracts of *A. calamus* against the third and fourth instar larvae of *Ae. aegypti* revealed that high percentage of larval mortality was observed at various concentrations as in Table 1. The result of regression analysis indicated that the mortality rate (Y) is positively correlated with the concentration (x) indicating mortality increased with increase in concentration (Figure 1). LC_{50} and LC_{90} of petroleum ether extract were 57.32 m+g/L and 120.13 mg/L respectively. LC_{50} and, LC_{90} of ethyl alcohol extract was 64.22 mg/L, 130.37 mg/L. Regression coefficient for petroleum ether ethyl alcohol extracts was close to one. *Chi*-square values were highly significant at $P<0.01$, degree of freedom and slope for petroleum ether and ethyl alcohol extracts is mentioned in Table 2. The obtained results revealed the LC_{50} of petroleum ether was less than the LC_{50} of ethyl alcohol so petroleum ether extract was consider more potent than ethyl alcohol extract. The probit regression line is plotted in Figure 2. With the help of this regression line regression equation and regression co-efficient were calculated (Figure 2).

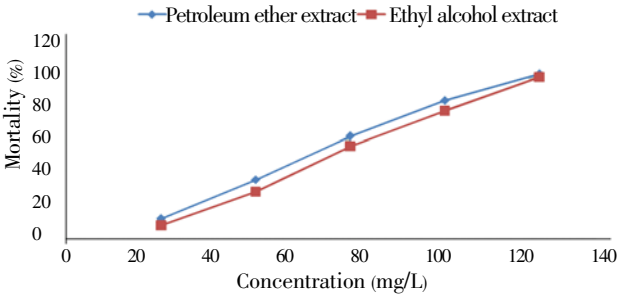

Figure 1. Dose response relationship for petroleum ether and ethyl alcohol extract of *A. calamus* applied for 24 h on 3rd and 4th instars larvae of *Ae. aegypti* L.

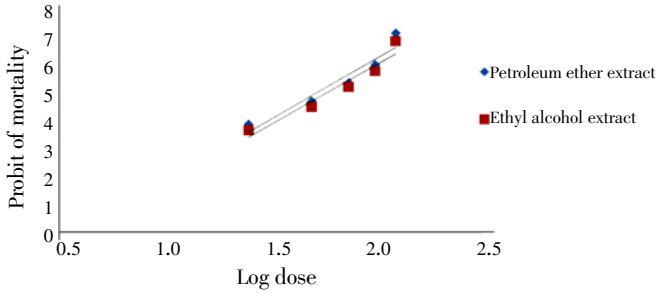

Figure 2. Larvicidal effects of petroleum ether and ethyl alcohol extract of *A. calamus* applied against 3rd and 4th instars larvae of *Ae. aegypti* L. expressed as linear regressions.

Table 1
Larvicidal activity of *A. calamus* extracts to the 3rd and 4th instar larvae of *Ae. aegypti* L.

Plant extracts	Observed mortality after 24 h (%)					
	25 mg/L	50 mg/L	75 mg/L	100 mg/L	125 mg/L	Control
A. calamus (Petoleum etherextract)	12%	35%	61%	82%	98%	0%
A. calamus (Ethyl alcohol extract)	8%	28%	55%	76%	96%	0%

Table 2
LC_{50} and LC_{90} with fiducial limits (95%) of tested plant extracts against larvae of *Ae. aegypti* L.

Plant material	LC_{50} (mg/L) (95% CL)	LC_{90} (mg/L) (95% CL)	χ^2	*df*	Slope±SE	Regression equation	R^2	*P* value
Acorus calamus (PE extract)	57.32 (40.77–73.99)	120.13 (89.49–244.30)	12.307	3	3.988±0.311	Y:4.2774x–2.4204	0.9149	0.006**
Acorus calamus (EA extract)	64.22 (47.95–81.78)	130.37 (98.11–258.97)	11.509	3	4.168±0.329	Y:4.2293x–2.5512	0.9339	0.009**

PE: Petoleum ether, EA: Ethyl alcohol, LC_{50}: Lethal concentration that kills 50% of the expose larvae, LC_{90}: Lethal concentration that kills 90% of the expose larvae. CL: Confidence limit, χ^2: *Chi*-square, *df*: Degree of freedom, SE: Standard error, Y: mortality rate, x: concentration, R^2: Regression co-efficient, **: highly significant at $P<0.01$ level.

4. Discussion

The present investigation revealed that the extract of *A. calamus* possess larvicidal activity against *Ae. aegypti* larvae. Crude extract of rhizome *A. calamus* showed effective result against third and fourth instar larvae of *Ae. aegypti*. Among the solvent extracts petroleum ether extract showed the best result against the mosquito larvae. Though several compounds of plant origin have been reported as larvicide[21], there is a wide scope for the discovery of more effective plant products. In fact many

researchers have reported on the effectiveness of plant extract against mosquito larvae. Chakkaravarthy *et al.*[9] reported the larvicidal efficacy of *Azadirachta indica* (A. Juss) and *Datura metal* (linn.) leaf extract against the third instar larva of *Culex quinquefasciatus* (Dipter: Culicidae) (*Cx. quinquefasciatus*). The hexane and chloroform extract shows LC_{50} values were 246.38, 198.82, 709.96 and 562.07 mg/L respectively. Kovendan K *et al.*[22] studied on *Orthosiphon thymiflorus*, the LC_{50} values of hexane, chloroform, ethyl acetate, acetone and methanol extract of *Orthosiphon thymiflorus* on third instar larvae of *Anopheles stephensi* were LC_{50}= 201.39, 178.76, 158.06, 139.22 and 118.74 mg/L; *Cx. quinquefasciatus* were LC_{50}=228.13, 209.72, 183.35, 163.55 and 149.96 mg/L and *Ae. aegypti* were LC_{50}=215.65, 197.91, 175.05, 154.80 and 137.26 mg/L respectively. Maximum larvicidal activity was observed in the methanolic extract followed by acetone, ethyl acetate, chloroform and hexane extract. As compared to the above study on plant extract our study showed lowest LC_{50} indicating highest larvicidal potential.

The larvicidal activities of different crude solvent extracts of benzene, hexane, ethyl acetate, methanol and chloroform leaf extract of *A. paniculata* was found to be more effective against *Cx. quinquefasciatus* than *Ae. aegypti*. The LC_{50} values were 112.19, 137.48, 118.67, 102.05, 91.20 mg/L and 119.58, 146.34, 124.24, 110.12, 99.54 mg/L respectively[23]. Maheshwaran *et al.*[24] reported solvent extracts of chloroform, ethanol and hexane, leaf extract of *Leucas aspera* against *Cx. quinquefasciatus* and *Ae. aegypti* 4th instar larvae and the LC_{50}values were 518.88, 1059.13, 193.43 and 588.76, 1565.95, 199.72 mg/L, respectively. Swathi *et al.*[25] evaluated ethanolic (ethyl alcohol) extracts of leaves of *Datura stramonium* for larvicidal and mosquito repellent activities against *Ae. aegypti*. The LD_{50} values for larvicidal activity were found to be 86.25 mg/L. Tarek M.Y. EL-Sheikh *et al.*[26] tested petroleum ether, ethanol and acetone extracts of leaves from the Egyptian plant *Cupressus sempervirens* (Cupressaceae) against 3rd instar larvae of the mosquito *Culex pipiens* L. LC_{50} values of ethanol, acetone and petroleum ether extract of *Cupressus sempervirens* were 263.6 , 104.3 and 37.8 mg/L respectively.

The extensive use of conventional synthetic insecticides results in environmental hazards and resistance in major species and this has necessitated the need to develop an alternate insecticide. Botanical insecticides provide an alternative to synthetic insecticides because they are generally considered safe, biodegradable, and can often be obtained from local sources. In addition, the use of medicinal plants for mosquito control is likely to generate local employment, reduce dependence and enhances public health.

The above mentioned researches are obviously worthy. However it is worth to note that their LC_{50} were much higher than the extracts which were tested in our study. The obtained results indicated that petroleum ether extract and ethyl alcohol extracts of *A. calamus* had the potential larvicidal efficacy but petroleum ether extract was more efficient than ethyl alcohol (ethanolic) extract. The larvicidal activity of rhizome may be due to the presence of the major chemical compound, β-asarone and limonene[27]. Therefore these results should encourage further studies on the identification of the active principles involved and their mode of action. Field trials are also needed to recommend *A. calamus* as an anti-mosquito product to combat and protect from mosquitoes in a control program.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Interests in *Aedes* mosquito lies in the fact that it acts as a vector for dengue fever and dengue hemorrhagic fever which is endemic in Southeast Asia, the Pacific Islands area, Africa and the Americas. Today, about two-fifths of the world's population is at risk for dengue, with cases reported in more than 100 countries. Indeed, the present recrudescence of these diseases is due to the higher number of breeding places in today's throwaway society and to the increasing resistance of mosquitoes to current commercial insecticides. Time and millions of money has been spent on researches on the dengue vaccine but nothing much is produced. Plants may be a source of alternative agents for control of mosquitoes because they are rich in bioactive chemicals, active against specific target-insects and are biodegradable. The present paper studied the therapeutic and pesticide properties of *A. calamus* because this plant is abundant in India.

Research frontiers

Study is being performed to determine the mosquito larvicidal activity of *A. calamus* plant. Compounds present in this plant are active against specific target-insects and are biodegradable.

Related reports

Many researchers have reported on the effectiveness of plant extract against mosquito larvae such as Chakkaravarthy *et al.* (2011), Kovendan K *et al.* (2013), Maheshwaran *et al.* (2008) and Swathi *et al.* (2012) who reported the larvicidal efficacy of plant extracts against the third and fourth instar larva.

Innovations & breakthroughs

The study have showed the mosquito larvicidal efficacy

of crude petroleum ether and ethanol extract of *A. calamus*. The study is highly important in the field of pharmacology for the development of novel drugs against mosquito in near future.

Applications

It is very interesting to see the utilization of commonly distributed plant (*A. calamus*) throughout the tropics and subtropics, especially in India and Sri Lanka for the extraction of novel bioactive compounds that possess unique importance and biomedical application. Thus, from this study it has been shown that the petroleum ether and ethanolic extract of *A. calamus* is pharmacologically important.

Peer review

The authors have evaluated the mosquito larvicidal activity of *A. calamus* for its biomedical application. Based on the results the authors have proposed that crude extracts of *A. calamus* showed good larvicidal activity. In general the article is well organized; materials and methods appear to be reproducible. The results of the present study are noteworthy and there is a high possibility of developing an ecofriendly phyto-insecticide.

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