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ORIGINAL PAPER

Larvicidal efficacy of *Adhatoda vasica* (L.) Nees against the bancroftian filariasis vector *Culex quinquefasciatus* Say and dengue vector *Aedes aegypti* L. in in vitro condition

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Abstract The larvicidal activities of methanolic fractions from *Adhatoda vasica* leaf extracts were investigated against the bancroftian filariasis vector *Culex quinquefasciatus* and dengue vector *Aedes aegypti*. The results indicated that the mortality rates was high at 100, 150, 200 and 250 ppm of methanol extract of fractions III with R_f value 0.67 and methanol extract of fraction V with R_f value 0.64 of *A. vasica* against all the larval instars of *C. quinquefasciatus* and *A. aegypti*. The result of log probit analysis (at 95% confidence level) revealed that lethal concentration, LC_{50} and LC_{90} values were 106.13 and 180.6 ppm for fraction III, 110.6 and 170 ppm for fraction V of *C. quinquefasciatus*. And, the LC_{50} and LC_{90} values were 157.5 and 215.5 ppm for fraction III of *A. aegypti* and 120 and 243.5 ppm for the fraction V of *A. aegypti*, respectively. All the tested fractions proved to have strong larvicidal activity (doses from 100 to 250 ppm) against *C. quinquefasciatus* and *A. aegypti*. In general, second instar was more susceptible than the later instar. The results achieved suggest that, in addition to their ethnopharmacology value, *A. vasica* may also serve as a natural larvicidal agent.

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Introduction

In the recent past, the use of synthetic chemical insecticides in mosquito control has resulted in ecological imbalance, mosquito resistance, the mosquito resurgence and increased toxicity to non-target organisms including natural enemies in the agro-ecosystems (Devine and Furlong 2007). Natural products of plant origin with insecticidal properties have gained importance in recent decades as one of the alternative means of mosquito control strategies, to minimize or eliminate the use of chemical pesticides (Kalaivani et al. 2011).

Mosquitoes are one of the most medically significant vectors that affects the health and well-being of humans and domestic animals, by transmitting parasites and pathogens. Malaria, dengue, yellow fever, filariasis, brain fever and chikungunya are some of the deadly diseases spread by mosquito vectors. *Culex quinquefasciatus* Say (Diptera: Culicidae) is a significant vector of bancroftian filariasis in tropical and subtropical regions. In India alone, 25 million people harbour microfilaria, and 19 million people suffer from filarial disease manifestations (NICD 1990; Maheswaran et al. 2008).

Aedes aegypti L (Diptera: Culicidae), a vector of dengue, is widely distributed in the tropical and subtropical zones. About two-thirds of the world's population lives in areas infested with dengue vectors. Dengue hemorrhagic fever occurs in Asia, the Americas and some pacific islands. Dengue is endemic in all continents except Europe, and epidemic dengue viruses, causative agents of dengue fever and more

severe dengue hemorrhagic fever or dengue shock syndrome infects over 100 million people every year (Hahn et al. 2001).

Recently, there has been a major effort to promote the use of botanicals as environmentally friendly pesticides, microbial sprays and insect growth regulators in combination with other control measures such as beneficial insects in an integrated control program (Ascher et al. 1995). Botanical insecticides have been used predominantly to control medicinal and veterinary important vectors like *Aedes*, *Anopheles*, and *Culex* (Amer and Mehlhorn 2006a, b, Anees 2008; Batabyal et al. 2009; Cetin et al. 2011); ticks: *Ixodes ricinus*, *Rhipicephalus sanguineus*, *Amblyomma;* fleas: *Ctenocephalides felis*; biting flies: *Stomoxys calcitrans* (Mehlhorn et al. 2005) and lice (Mehlhorn et al. 2011) during the last two decades.

Adhatoda vasica (L.) Nees (Acanthaceae) is a shrub. Its leaves are simple, and flowers are white, pink or purple. The medicinal properties of *A. vasica* are well-known in India and several other countries for many years. The leaves contain an essential oil and the alkaloids, quinazoline, vasicine, vasicinone and deoxyvasicine (Chowdhury and Bhattacharyya 1985; Claeson et al. 2000). The roots contain vasicinolone, vasicol, peganine and 2′-hydroxy-4-glucosyl-oxychalcone. The flowers contain D-glucoside, kaempferol and its glucosides, as well as the bioflavonoid, namely quercetin (Srivastava et al. 2001).

The exploit of *A. vasica* extract, in contrast to the agriculturally significant pest, was well proven (Sadek 2003) but the action against the medicinally important vector is not clearly established. Hence, an effort has been made to determine the effects of, *A. vasica* fractions on the larval mortality of filariasis and dengue vector.

Materials and methods

Mosquito culture

C. quinquefasciatus and *A. aegypti* culture has been maintained at the Biopesticides and Environmental Toxicology Laboratory (BET Lab), SPK Centre for Excellence in Environmental Sciences since at least 2007, without exposure to pesticides. They were maintained at 27±2°C and 75–85% RH under a 14:10 L/D photoperiod. Larvae were fed a diet of Brewer's yeast, dog biscuits and algae collected from ponds in a ratio of 3:1:1, respectively. Pupae were transferred from the trays to a cup containing to tap water and placed in screened cages (23×23×32 cm) where adults emerged. Adults were maintained in 30×30×30-cm glass cages. Adults were continuously provided with 10% sucrose solution in a jar with a cotton wick. On day 5, post-emergences, adults were deprived of sugar for 12 h, then provided with a mouse placed in resting cages overnight for blood feeding by females. Adult mosquitoes were maintained under the same environmental conditions as the larvae (Kalaivani et al. 2011).

Methanolic extracts of leaves of *A. vasica*

The leaves of *A. vasica* were collected from trees of natural forests of Kolli hills, Namakkal District, Tamil Nadu during morning hours (Fig. 1). Extracts of leaves were obtained according to the following methodology. First, the plant leaves were crushed to fine particle size and dried in an oven at 35ºC for 20 h. Extraction was carried out according to the procedure of Warthen et al. (1984). In a 1,000-ml flask, 100 g of crushed and dried plant materials in 1000 ml of methanol were stirred for 3 h. After leaving the methanolic solution to rest overnight, it was filtered through Whatman no. 40 filter paper (Whatman International Ltd., Maidstone, England). The solid filtration residue was extracted again following an identical procedure, and the two filtrates were combined. The solvent was removed by vacuum evaporation in a rotary evaporator; dark-green colour extracted from leaves was obtained. That crude extract was used to prepare a stock solution (Senthil-Nathan et al. 2006a, b).

Chromatographic analysis

The excessive solvent in each partitioned fraction was removed by a rotary evaporator. The soluble fraction was further divided into 20 subfractions by liquid chromatography in an open column with silica gel 60 (Lobachemie). The column was eluted with gradient benzene and methanol from 98/2 to 0/100. The dried subtractions were grouped by thin layer chromatography (TLC) with silica gel 60 coatings. TLC fingerprint profiles of all the fractions of *A. vasica* were developed in the solvent system of chloroform/benzene/acetic acid (3:2:1; at 25±2°C temperature and 40% relative humidity) and Co-TLC of all the fractions was carried out.

Fig. 1 External morphology of *A. vasica*

Larvicidal assay

Bioassays were performed on first to fourth instars of *C. quinquefasciatus* and *A. aegypti* using concentrations of 100, 150, 200 and 250 ppm *A. vasica* subfraction extract. Methanol (0.1%) served as a control. A minimum of 20 larvae per concentration was used for all the experiments, and the experiments were replicated five times (total, n= 100). The lethal concentration (LC_{50} and LC_{90}) was calculated using probit analysis (Finney 1971). For mortality studies, 20 larvae each of second, third and fourth instars were introduced to a 250-ml glass beaker containing various concentrations of the *A. vasica* subfraction extract supplemented with 50 mg/l of yeast extract. A control was also maintained with methanol. The treatments were replicated five times, and each replicated set contained one control. Percentage mortality in the treatments was corrected when necessary for mortality in the controls using Abbott (1925) formula

$$\text{Percentage of mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

Statistical analysis

Data from mortality experiments were subjected to analysis of variance (ANOVA of arcsine, logarithmic and square root transformed percentages). Differences between the treatments were determined by Tukey's multiple range test (P= 0.05; Snedecor and Cochran 1989). The relationship between probit and log concentrations were established as probit equations and probit regression lines were drawn for each of larval stage.

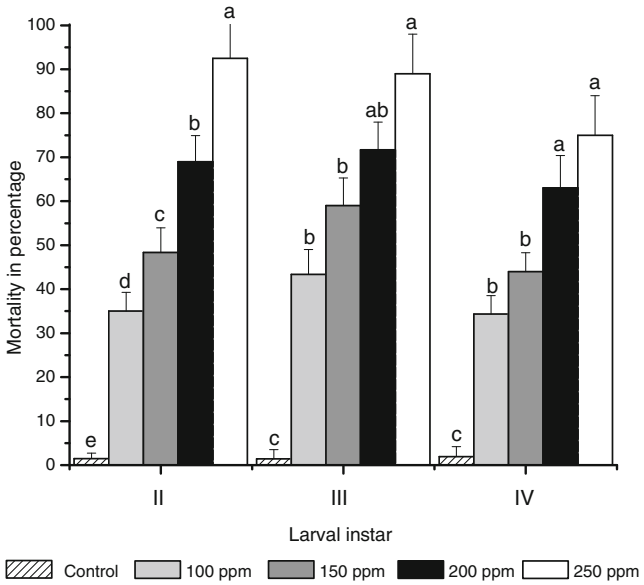

Fig. 2 Percentage mortality of the fraction III of *A. vasica* against second, third and fourth larval instar of *C. quinquefasciatus*. Mean (±SEM) followed by the *same letter* in individual larval instar in *bars* indicate no significant difference (P<0.05) in a Tukey's test

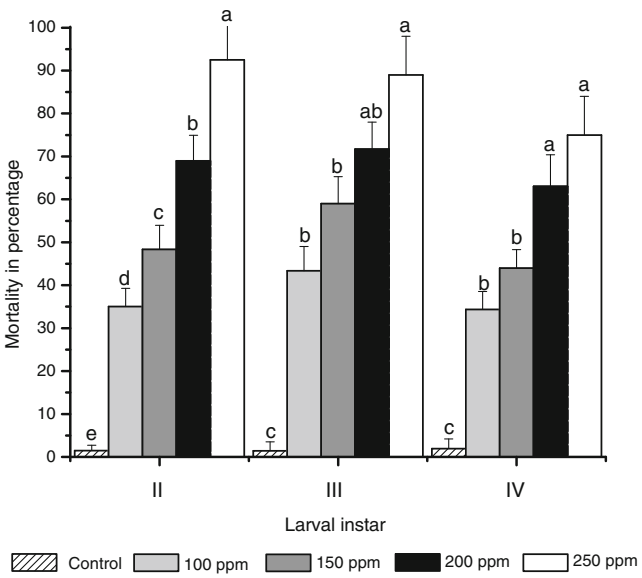

Fig. 3 Percentage mortality of the fraction V of *A. vasica* against second, third and fourth larval instar of *C. quinquefasciatus*. Mean (±SEM) followed by the *same letter* in individual larval instar in *bars* indicate no significant difference (P<0.05) in a Tukey's test

Results

In the present study, the toxicity of *A. vasica* was tested against four different larval stages of *C. quinquefasciatus*

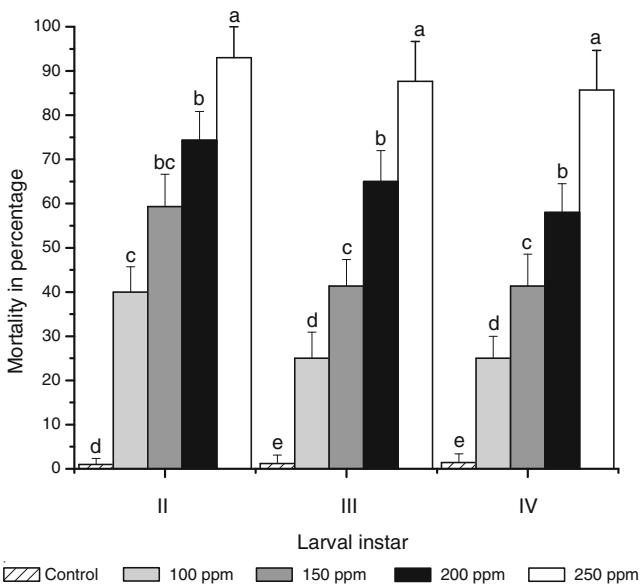

Fig. 4 Percentage mortality of the fraction III of *A. vasica* against second, third and fourth larval instar of *A. aegypti*. Mean (±SEM) followed by the *same letter* in individual larval instar in *bars* indicate no significant difference (P<0.05) in a Tukey's test

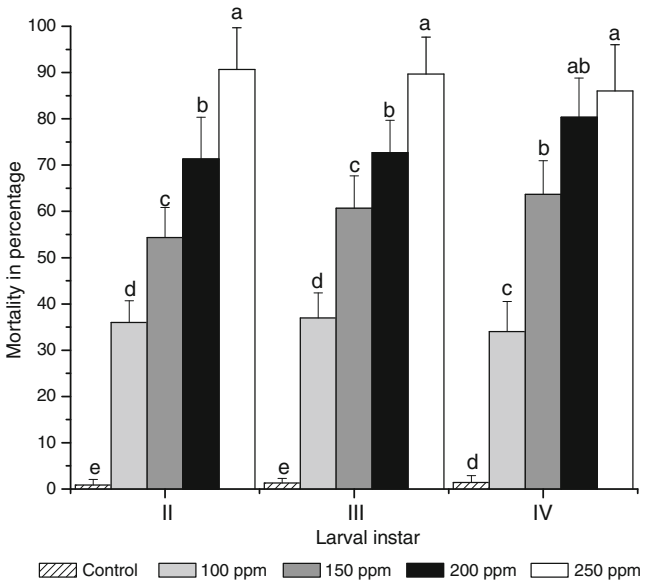

Fig. 5 Percentage mortality of the fraction V of *A. vasica* against second, third and fourth larval instar of *A. aegypti*. Mean (±SEM) followed by the *same letter* in individual larval instar in *bars* indicate no significant difference (P<0.05) in a Tukey's test

and *A. aegypti*. The data were recorded and statistical data regarding LC_{50} and LC_{90} were calculated. Exposure of leaf extracts in the mosquito larval diet increased mortality in all larval instars. The effect on larval mortality was dependent on concentration. Leaf extracts were potent in all experiments with least LC_{50}. It is clearly pointed out that the high concentration of the fractions from *A. vasica* plant extracts produced high mortality in the initial larval stages. The most potent *A. vasica* extract with an LC_{50} of 120 and an LC_{90} of 243.5 ppm, after 24 h. The survival of the larvae was significantly reduced by all the formulation treatments with 100, 150, 200 and 250 ppm of *A. vasica*.

At the concentrations of 200 ppm of the fraction III with R_f value 0.67 of *A. vasica* formulation, over 80% of the observed mortality occurred within the first 24 h (F=43.49; df=4; P<0.001 for second instar, F=37.59 df=4; P<0.001 for third instars and F=45.39; df=4; P<0.001 for fourth instars larvae for *C. quinquefasciatus* and F=42.48; df=4; P<0.001 for second instar, F=41.7; df=4; P<0.001 for third instar and F=43.0; df=4; P<0.001 for fourth instar larvae of *A. aegypti*) and fraction V with R_f value 0.64 of *A. vasica* formulation, over 80% of the observed mortality occurred within the first 24 h, (F=29.57; df=4; P<0.001 for second instar, F=18.08; df=4; P<0.001 for third instar and F=27.57; df=4; P<0.001 for fourth instar larvae for *C. quinquefasciatus* and F=36.04; df=4; P<0.001 for second instar, F=42.74; df=4; P<0.001 for third instar and F=45.57; df=4; P<0.001 for fourth instar larvae of *A. aegypti*). In lower concentrations, the rate of mortality was very slow, and some larvae lived with deformed structure. Thus, lethal effects on early larval instars appear to greatly reduce survival of later instars. Second instar larvae were most susceptible in bioassay experiments with the lowest lethal concentrations. Figures 2, 3, 4, and 5 showed that the mortality rate of the *A. vasica* formulation was higher in all tested concentrations.

The obtained results revealed the larvicidal effect of *A. vasica* fraction III on *A. aegypti* and *C. quinquefasciatus*. The highest larval mortality was found in methanol extract of *A. vasica* with LC_{50} of 56.13 ppm and LC_{90} of 130.6 ppm against fourth instar larvae (Fig. 6) of *C. quinquefasciatus*. Also, the LC_{50} of 157.5 ppm and LC_{90} of 215.5 ppm were observed and against fourth instar larvae of *A. aegypti*, respectively (Figs. 6, 7, 8, and 9). The results pointed out that the mortality rates at 100, 150, 200 and 250 ppm of fractions III with R_f value 0.67 and fraction V with R_f value 0.64 of *A. vasica* concentrations were highest among all concentrations of the extracts tested against all the larval instars (Fig. 10).

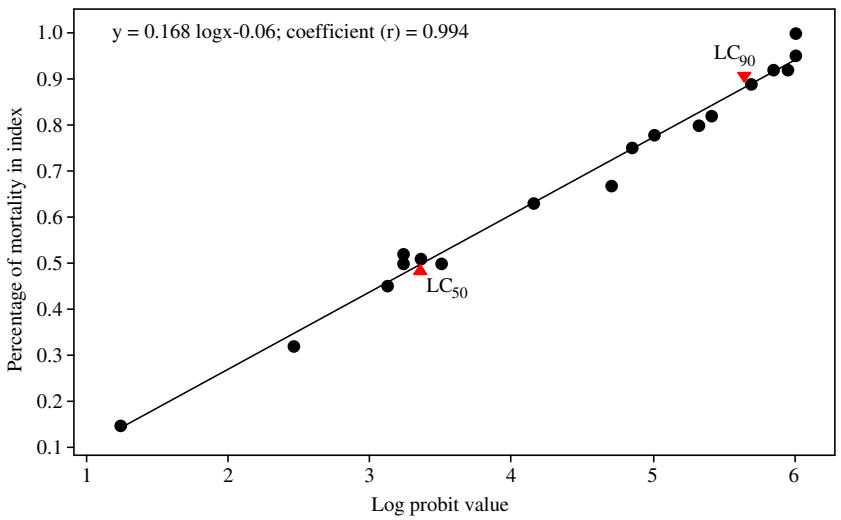

Fig. 6 Lethal concentrations (LC_{50} and $_{90}$) of *A. vasica* fraction III against *C. quinquefasciatus*

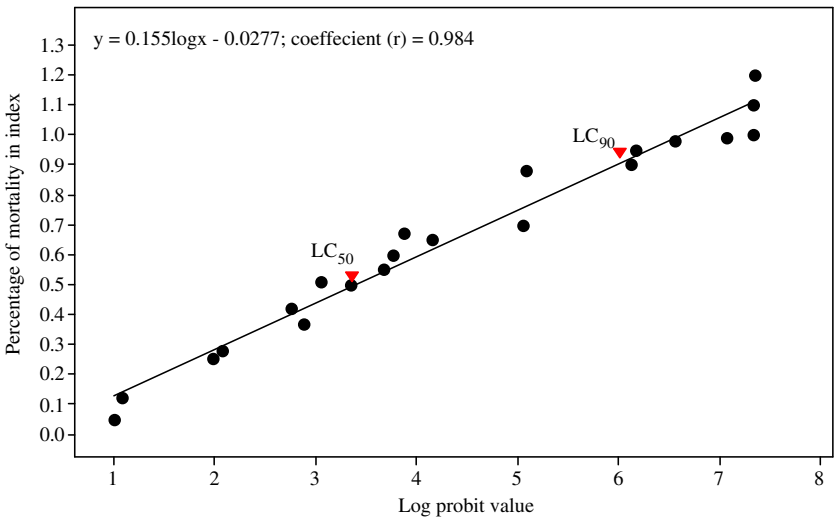

Fig. 7 Lethal concentrations (LC_{50} and $_{90}$) of *A. vasica* fraction V against *C. quinquefasciatus*

Discussion

The fractions obtained from the *A. vasica* were an effective larvicidal agent against the *C. quinquefasciatus* and *A. aegypti* larvae. It was highly toxic to mosquito larvae and also inhibited the development of pupae. The high rate of larval mortality of mosquitoes observed at higher concentrations (250 ppm of *A. vasica*) within a 24-h exposure indicates the high toxicity of the product. The plants tested in the present study are reported to be eco-friendly and toxic to agriculturally important insect pest (Sadek 2003). It is clearly proven that crude or partially purified plant extracts are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extracts (Koleva et al. 2002; Senthil-Nathan 2007; Senthil-Nathan et al. 2006a, b)

The partially purified fraction of *A. vasica* leaves was found to have apparent toxic effects on the larvae of both filariasis and dengue vector. The mortality experiments showed that the test larvae had an irregular movement suggesting that the reduced survivability. The fraction of *A. vasica* leaves exhibited chronic toxicity against *C. quinquefasciatus* and *A. aegypti* larvae. The toxicity was initially showed by the mortality of all larvae reared on treated water, and was further demonstrated in the strikingly low survival rates observed among all treatments containing high concentrations of the fractions from the *A. vasica* extract.

The toxic effects of *A. vasica* fraction from leaf and root extract studied in the present study and other studies (Bhaduri et al. 1985; Hiremath et al. 1997; Sadek 2003; Lateef et al. 2003; Al-Shaibani et al. 2008; Govindappa et al. 2011) can be referred to one or more of the many bioactive compounds included in the plant leaves. Among *A. vasica* leaf compounds that may interact with the feeding activity of insects are flavonoids and terpenoids (Rahman et al. 1997), hydroxyketones (Singh et al. 1991) and alkaloids (Chowdhury

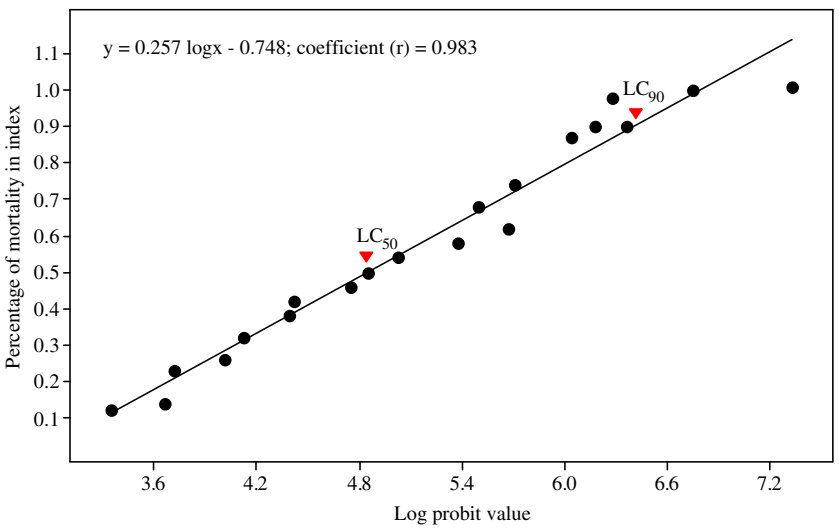

Fig. 8 Lethal concentrations (LC_{50} and $_{90}$) of *A. vasica* fraction III against *A. aegypti*

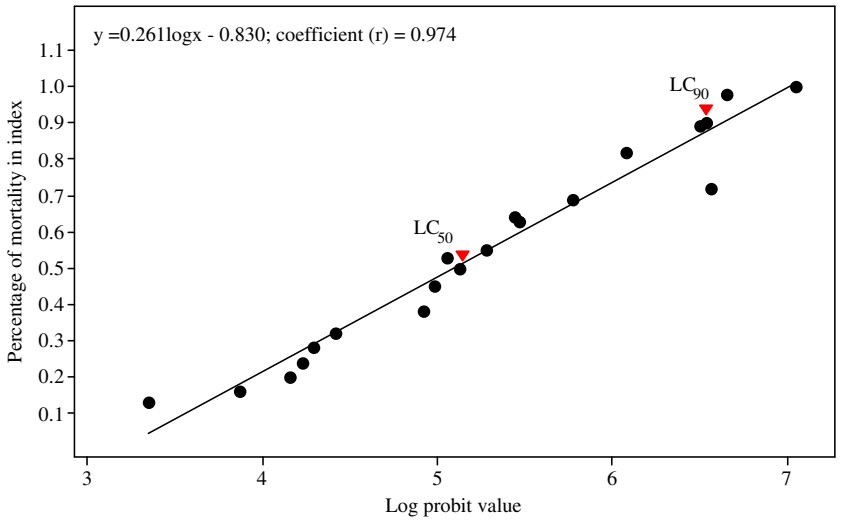

Fig. 9 Lethal concentrations (LC_{50} and $_{90}$) of *A. vasica* fraction V against *A. aegypti*

and Bhattacharyya 1985; Thappa et al. 1996; Srivastava et al. 2001). Screening the effects of five alkaloids isolated from *A. vasica* leaves against four insect species, Saxena et al. (1986) found that the effects vary in extent and type (i.e. being toxic, feeding deterrent or sterilizing) depending on the compound and the species involved.

Further, Al-Shaibani et al. (2008) found the aqueous and ethanolic extracts at 25–50 mg/ml concentrations displayed ovicidal and larvicidal activity against sheep gastrointestinal nematodes, *Haemonchus contortus*, *Trichostrongylus* spp., *Ostertagia circumcincta*, *Strongyloides papillosus*, *Oesphagostomum columbianum*, and *Chabertia ovina*. Furthermore, Lateef et al. (2003) conducted analogy studies on anthelmintic evaluation of *A. vasica* root extract against gastrointestinal nematodes, *H. contortus* of sheep in vitro and in vivo through the adult motility and faecal egg count reduction test, respectively and stated that methanolic and aqueous extracts of *A. vasica* displayed anthelmintic activity on adult worms in vitro and in vivo.

In a recent study carried out by Govindappa et al. (2011), *A. vasica* leaf extract considerably reduced the growth of bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* in vitro. Seed treatment was also found to be efficient in reducing the prevalence of the disease under the greenhouse conditions. Physiological observation of *A. vasica*-treated plants indicated that restriction of disease development in plant tissue. In addition, they proved that the *A. vasica* leaf extract has the ability to induce the activation of defense enzymes accumulation, which can be associated with induction of resistance against rice bacterial leaf blight. The present study shows that the fractions of *A. vasica* leaves extract have a strong toxic activity against both vectors. It is due to the fact that the array of compounds in the plant leaves is responsible for the acquired results.

Recently, biopesticides with plant origins are given for use against several insect species, particularly disease-transmitting vectors, based on the fact the compounds of plant source are safer in usage, without phytotoxic properties; also leave no scum in the environment (Schmutterer 1990; Senthil-Nathan et al. 2005; 2008). The results of the present research showed that *A. vasica* has good larvicidal activity against *C. quinquefasciatus* and *A. aegypti* .

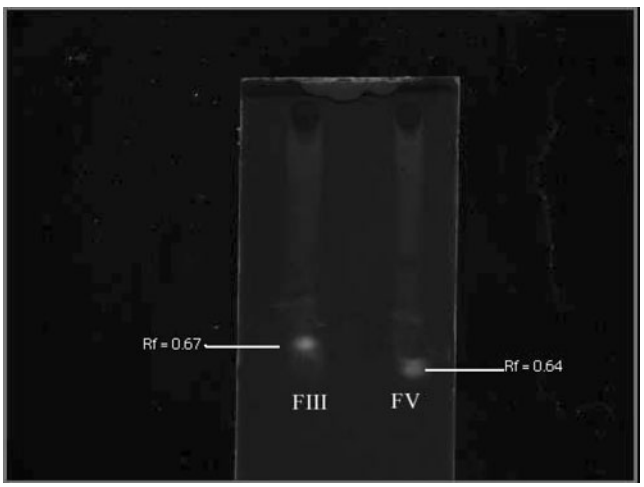

Fig. 10 A thin layer chromatography (TLC) fractions of *A. vasica* methanol extract

Conclusions

In conclusion, fractions of *A. vasica* plant extract produced more than 90% mortality of all instars of *C. quinquefasciatus* and *A. aegypti* at concentrations of 100, 150, 200 and 250 ppm. The purified fractions of *A. vasica* may be used as eco-friendly and sustainable insecticides to control mosquito vectors.

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