ENTOMOLOGY

Larvicidal activity of Vietnamese *Solanum nigrum* on mosquitoes *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae)

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Abstract

A Vietnamese domestic plant namely *Solanum nigrum* (*S. nigrum*) was subjected to test for larvicidal activity on two majors Dengue hemorrhagic fever (DHF) vectors *Aedes aegypti* (*Ae. aegypti*) and *Aedes albopictus (Ae. albopictus*). The plant was processed to get infusions in hot water or extracted in ethanol. Laboratory and field larval strains of two *Aedes* species were exposed to the infusions and extract at increasing concentrations for one hour and followed-up intensively for up to 72 hours. The obtained results of bioassay showed larvicidal effects of extract on all mosquito strains. The effects on laboratory strain of *Ae. aegypti* larvae were correlated with infusions and extract concentrations. Chopped plant infusions in hot water indicated mortality up to 77.3% of larvae. Ground plant infusions killed all of exposed larvae at day 3 post-exposure. Median lethal concentrations (LC50,s) of chopped and ground plant infusions were 10.25 and 7.54%, respectively. Ethanolic extract had very strong effect on experimental subjects. Within 72 hours, 100% of laboratory strain of *Ae. aegypti* larvae died after exposure to extract at 100 parts per million (ppm) or higher concentrations. Ethanolic plant extract showed similar larvicidal effect on field strains of *Ae. aegypti* and *Ae. albopictus*. The percentage mortality of field strains larvae reached 100% after exposure to 100 ppm of plant extract. At concentrations of 1000 ppm, 100% of exposed larvae died with 8 hours. LC50 on tested larvae was 25.07-33.60 ppm. Strong larvicidal activity of *S. nigrum* suggests the possible application in DHF vector control effort.

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Introduction

Aedes aegypti (Ae. aegypti) and *Aedes albopictus (Ae. albopictus)* are important vectors and widely distributed in the world including Vietnam. This mosquito can transmit some flaviviruses, such as Dengue, Chikungunya, Zika and yellow fever viruses (Tilak *et al*., 2016). Amongst the mentioned pathogens, Dengue virus is the most dangerous one. The habitat of *Ae. aegypti* is human accommodation (anthropophilic behavior) and the lack of effective vaccines against the virus are causes of deadly DHF outbreaks. There are some 50 million people infected with Dengue virus and a half million DHF cases needed treatment in hospitals each year (Rodriguez-Roche and Gould, 2013). In addition to life-threat to those who acquired the virus and highly cost for treatments, DHF also requires man power for nursing care and substitution for in-patients (Lee *et al*., 2017).

The main vector of DHF is attributed *Ae. aegypti* which is following by *Ae. albopictus* (Whitehorn *et al*., 2015). In Vietnamese urban areas, characteristic life styles of residents and architect of houses are favorable conditions for infestation of both mosquito species (Tsuzuki *et al*., 2013). These vectors could even survive rigorous winters in Northern Vietnam by taking advantage of human constructions to avoid cold temperature as low as 8.5°C (Tsunoda *et al*., 2014). As the consequence, the country was always in DHF endemic status with about more than 90,000 cases per year were reported (Hung *et al*., 2018). Unfortunately, there is neither specific treatment nor vaccine for DHF at the moment, so the best prevention way is the interruption of human and vector interaction. Current popular vector control methods are topical repellency, insecticide spray, ovitrap, and larvicidal application. The use of mosquitocidal chemicals is the first choice to prevent arbovirus dissemination in communities but also result in multiple resistance (Aponte *et al*., 2018). Another problem of spraying chemicals to kill mosquitoes is environmental pollution. Extremely high concentrations of airborne insecticides in Hanoi, Vietnam were considered the result of intensive use of spaying chemicals when DHF outbreak occurred in 2017 (Duong *et al*., 2018).

Current popular vector control methods are topical repellency, insecticide spray, ovitrap, and larvicidal application. Results from some vector control trials showed that larvicidal approaches were very promising (Williams *et al*., 2007 and Hall *et al*., 2017). The most common larvicidal reagents are synthetic chemicals because of their cost and effectiveness. However, mosquito control practice depends on not only scientific method but also on attitude and acceptance of populations. The campaigns to control mosquitos in DHF outbreaks in Vietnam sometimes faced non-cooperation from residents because of the fear of chemicals. Moreover, the limited budget for reagents and man power is a hamper against vector control effects. This fact leads to the need of simple and low cost methods which can be accepted and accessible to people. Although the efficacy of phytochemicals against mosquito larvae depends on plant species, plant parts, seasons, solvents and extraction methods, larvicidal effect of some plants has been approved effective in some tropical countries in South hemisphere (de Omena *et al*., 2007 and de la Torre Rodriguez *et al*., 2013). Several species of tested plants are also found in Vietnam, such as *S. nigrum, Operculina pterripes*… Crude and solvent extracts of *S. nigrum* berries were able to kill *Culex quinquefasciatus* larvae (Rawani *et al*., 2013). In addition to extract from berries, larvicidal activity against *Ae. aegypti* of crude extract from *S. nigrum* was also approved (Patel *et al*., 2018). The evidence of larvicidal effects of *S. nigrum* suggests potential practical application in vector control, particularly in DHF prevention. However, we did not know if the plants grown in Vietnam have similar effects on local mosquitoes as seen elsewhere in the world. In DHF vector control tasks, involvement of households is very important and effective. So another question was if households could process the plants at homes without losing the desire effects. In this study, we carried some methods to extract *S. nigrum* and tested its larvicidal properties on laboratory and field strains of *Ae. aegypti* and *Ae. Albopictus*.

Materials and Methods

Plant collection

The healthy mature plants of *S. nigrum* ("Lu lu duc", "Ca lu", or "Thu lu duc" in Vietnamese langue) were harvested in Thanh Phong commune, Thanh Liem district, Ha Nam province, Vietnam. The plants were authenticated by plant specialists from Institute of Ecology and Biological resources. Voucher specimens were deposited at the Department of Parasitology and Entomology, Vietnam Military Medical University (VMMU).

Maintenance of mosquito strains

Two strains of *Ae. aegypti* (laboratory and Hue strains) and two strains of *Ae. albopictus* (Dak Lak and Hue strains) were used in this study. The laboratory strain of *Ae. aegypti* was a kindly gift from Dr. Vu Duc Chinh (Department of Entomology, National Institute of Malaria, Parasitology and Entomology, Vietnam). This strain has been maintained for about forty years, was sensitive to common insecticides and was usually used as a reference for insecticide susceptibility studies. The other mosquitoes were field strains in Vietnam, came from larvae collected in highland province of Dak Lak (Dak Lak strains) and central province of Thua Thien – Hue (Hue strains) in a Dengue vector surveillance in August and September, 2018. The distance of locations where field strains were collected is about 600 kilometers. When experiments were carried out, field mosquitoes have been proliferated for 3-5 generations.

Mosquitoes were maintained in 30 cm x 30 cm cages, in the insectarium, Department of Parasitology and Entomology, VMMU, at 27±2°C, 70-90% relative humidity, with photo period of 14-hour light and 10-hour dark. Mosquitoes fed on cotton balls soaked with 5% of saccharose and multi-vitamin supplement. After mating, female mosquitoes were offered blood meals from white mice three times a week. The female mosquitoes were allowed to lay eggs on white filter papers lined on inner walls of plastic bowls containing dechlorinated tap water. Egg papers were collected every three days, dried and kept in plastic bags, or were hatched to amplify the numbers of larvae and mosquitoes. Larvae were risen in 15 cm x 25 cm x 8 cm plastic boxes containing water with 2-cm depth and fed on fish pellets.

Preparation of chopped plant infusion

The whole plants were washed gently three times and dried at room temperature for 24 hours. The plants were chopped into pieces of 1 cm, then mixed 100 grams of plant pieces with 500 mL of boiling water. Let the water cooled down at room temperature for about 2 hours. This water was called 20 % chopped plant infusion (CPI) and kept at 4°C. CPI was diluted in dechlorinated tap water to get 1.25%, 2.5%, 5%, 10% and 20% infusion for larvicidal bioassay.

Preparation of ground plant infusion

The whole plants were washed gently three times and dried at room temperature for 24 hours. The plants were ground in a blender. Mixed 100 grams of ground plant with 500 mL of boiling water. This soup was cooled down at room temperature for about 2 hours. Then removed plant debris from the soup by filter papers. This soup was called 20 % ground plant infusion (GPI) and kept at 4°C. GPI was diluted in dechlorinated tap water to get 1.25%, 2.5%, 5%, 10% and 20% infusion for larvicidal bioassay.

Preparation of plant extract

The whole plants were washed gently three times, chopped into pieces of 1 cm and dried at room temperature for 10 days. The mass of plants decreased by about 82% after dehydration. Immerged 2 kilograms of dried plant in 4 liters of absolute ethanol for 5 days. Passed ethanol infusion through filter papers to remove debris. Ethanol was then eliminated by using a vacuum rotator (Yamato Rotary RE300), at 31 revolutions per minute, -15 bar. After ethanol evaporation completed, 16.4 grams of dark viscous plant extract was collected and kept at 4°C. Diluted the plant extract in dimethyl

sulfoxide (DMSO) to reach final concentration of 200 mg plant extract in one mL. The stock extract solution of 200 mg/mL in DMSO was further diluted in water and DMSO to get final plant extract concentrations of 20, 40, 80, 100, 200, 400, 800 and 1000 ppm. DMSO was adjusted so that the final DMSO concentrations were fixed at 2%.

Larvicidal bioassay

Infusions and extract of *S. nigrum* for larvicidal tests were prepared as mentioned above. Tap water was used as control for infusions and 2% of DMSO in water was negative control in the tests with plant extract. The tests were done in 150 mL plastic cups. Each cup was added 50 mL of infusions or extract at different concentrations. Then 25 second or third larval instars were transferred into the cups. The larvae were exposed to the test infusions or extract for 24 hours, then alive larvae washed 3 times in tap water and finally reared according to regular maintenance protocol.

Each experiment was repeated three times.

Data analysis

The data were analyzed using GraphPad Prism 8.0 software. Lethal concentration 50% (LC50) was calculated according to probit analysis (Finney, 1971).

Results

Effects of *S. nigrum* infusion and extract on laboratory *Ae. aegypti* strain

Ae. aegypti larvae of aboratory strain were challenged with infusion and ethanolic extract of *S. nigrum* to figure out the plant processing method giving highest larvicidal activity. Amounts of fresh plant to make 100 mL of test solution at different concentrations were shown in Tables 1-3.

Larvicidal activity of S. nigrum chopped infusion in hot water on Ae. aegypti, laboratory strain

Effects of *S. nigrum* CPI from hot water on laboratory strain larvae of *Ae. aegypti* were present in Figure 1. After 72 hours of exposure to 1.25%, 2.5%, 5%, 10% and 20% CPI, the percentages of death larvae were 2.67%, 5.33%, 12.0%, 44.0% and 77.3%, respectively. This means that 1.25% CPI had no effect to the larvae according to the comparison to negative control. But when the concentrations increased, their larvicidal effects changed in a coordinate fashion. 5% CPI reached maximal effect at 32 hours post-exposure. The number of died larvae by 10% and 20% CPI increased sharply and almost reached highest activity within the first day. LC50 of *S. nigrum* CPI was 10.25% (Figure 1B).

Table 1. Amounts of plant needed to make 100 mL of plant infusions.

Chopped plant infusions		Ground plant infusions	
Concentrations (%)	Plant (Grams)	Concentrations (%)	Plant (Grams)
20	20	20	20
10	10	10	10
5	5	5	5
2.5	2.5	2.5	2.5
1.25	1.25	1.25	1.25

Table 2. Components of plant ethanolic extract.

Components	Extract concentrations (ppm)								
	0	20	40	80	100	200	400	800	1000
Stock extract (mL)	0.0	0.01	0.02	0.04	0.05	0.1	0.2	0.4	0.5
DMSO (mL)	2.0	1.99	1.98	1.96	1.95	1.9	1.8	1.6	1.5
Water (mL)	98	98	98	98	98	98	98	98	98
Total volume (mL)	100	100	100	100	100	100	100	100	100

Table 3. Amounts of plant needed to make 100 mL of diluted extracts

Concentrations	Extract (Grams)	Dried plant (Grams)	Fresh plant (Grams)
Stock (200 mg/mL)	20	2439.02	13550.14
1000 ppm	0.1	12.20	67.75
800 ppm	0.08	9.76	54.20
400 ppm	0.04	4.88	27.10
200 ppm	0.02	2.44	13.55
100 ppm	0.01	1.22	6.78
80 ppm	0.008	0.98	5.42
40 ppm	0.004	0.49	2.71
20 ppm	0.002	0.24	1.36

Larvicidal activity of S. nigrum ground infusion in hot water on Ae. aegypti, laboratory strain

Figure 2 showed the activity of *S. nigrum* infusion from ground plant on laboratory strain larvae. The percentage of death larvae were 1.33%, 5.33%, 8.0%, 88.0% and 100% after 72 hours of exposure to 1.25%, 2.5%, 5%, 10% and 20% GPI, respectively (Figure 2A). Similarly to CPI, 1.25% of GPI had no effect to the larvae according to the comparison to control. The effects of higher concentrations showed that the stronger solution gave stronger larvicidal activity. As same as infusion from chopped

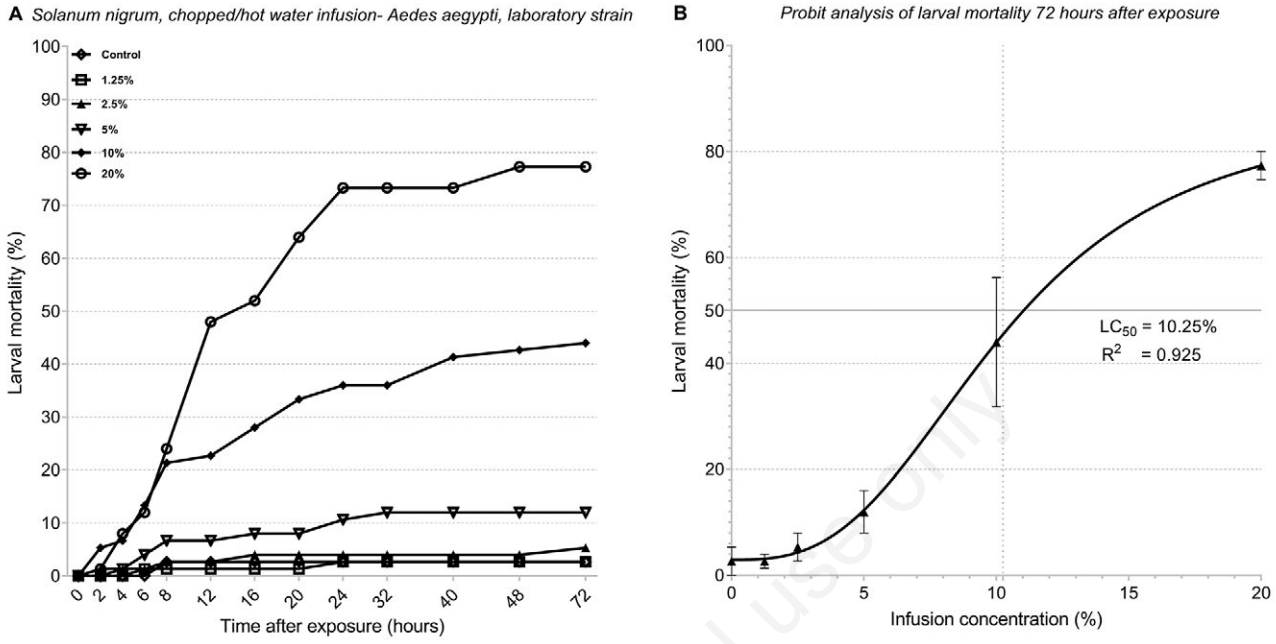

Figure 1. Larvicidal activity of chopped *S. nigrum* infusion on *Ae. aegypti*, laboratory strain. *S. nigrum* was chopped and infused in boiling water. Larvae were exposed to 1.25 – 20% infusions for 1 hour and followed up for 72 hours. (A) Numbers of dead larvae at different time points were recorded. (B) LC50 determination by probit analysis of larval mortality 72 hours after exposure. Each bioassay was repreated 3 times.

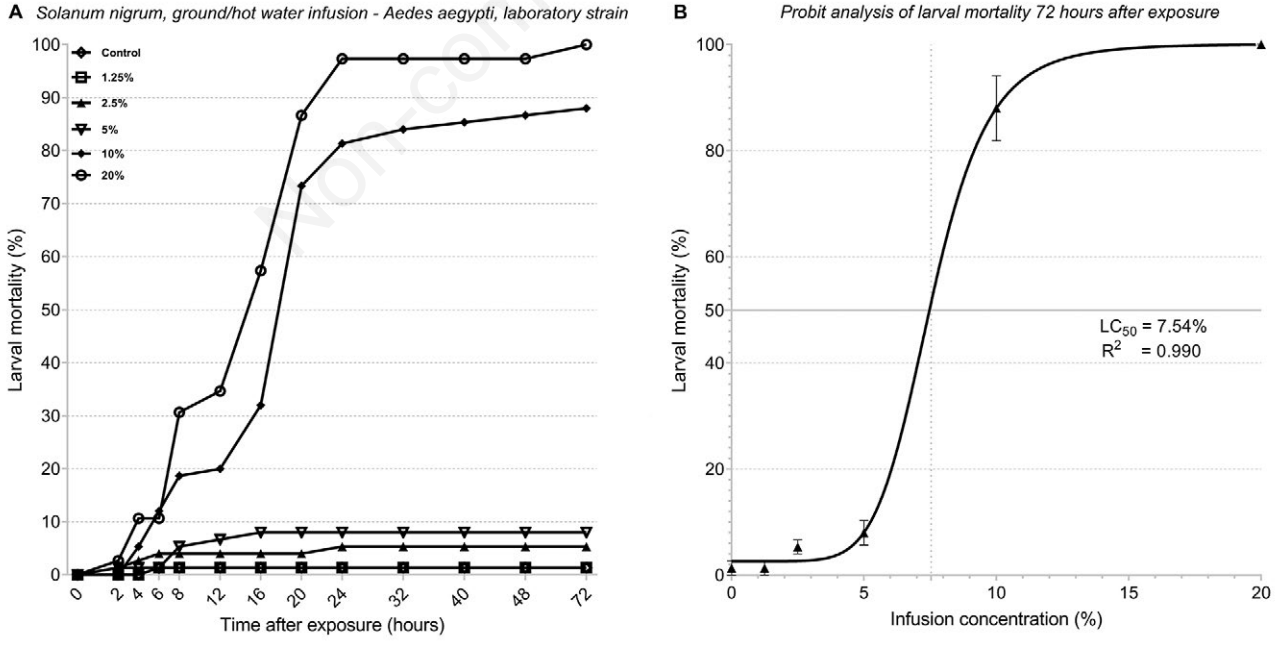

Figure 2. Larvicidal activity of ground *S. nigrum* infusion on *Ae. aegypti*, laboratory strain. *S. nigrum* was ground and infused in boiling water. Larvae were exposed to 1.25 – 20% infusions for 1 hour and followed up for 72 hours. (A) Numbers of dead larvae at different time points were recorded. (B) LC50 determination by probit analysis of larval mortality 72 hours after exposure. Each bioassay was repreated 3 times.

plants, the infusion from ground plants at concentrations of 10% and 20% had very strong activity in the 1st day. All of larvae died after 3 days post-exposure to 20% GPI with LC50 of 7.54% (Figure 2B).

Larvicidal activity of S. nigrum *ethanolic extract on Ae. aegypti laboratory strain*

S. nigrum extract showed very strong larvicidal activity on laboratory strain larvae (Figure 3). There was no death in the negative control group (Figure 3A). In the group treated with the lowest concentration of 20 ppm, there was no dead in the first 20 hours, the percentage of larvae died after 3 days was 1.33%. When the extract concentration was doubled to 40 ppm and quadrupled to 80 ppm, the number of larvae died increased dramatically to 76% and 80%, respectively. At higher extract concentrations (100, 200, 400, 800 and 1000 ppm), all of larvae were killed within 48 hours. LC50 of *S. nigrum* ethanolic extract on *Ae. aegypti* laboratory strain was 33.60 ppm (Figure 3B).

Effects of *S. nigrum* ethanolic extract on wild strains of *Ae. aegypti* and *Ae. albopictus*

Larvicidal activity of S. nigrum extract on Hue strain of Ae. aegypti

After bioassay tests on laboratory *Ae. aegypti*, the extract of *S. nigrum* showed very promising effect on the larvae. However, the laboratory mosquitoes were sensitive to mosquitocidal chemicals thus they were usually used as a control for insecticide susceptibility. It was unknown that the plant had the same effects on field mosquitoes. To clear this question, we decided to carry on bioassay tests on *Ae. aegypti* strains collected from Hue and *Ae. albopictus* from Hue and Dak Lak. Because of dominant effect of ethanolic extract, we skipped infusions and tested only the plant extract.

Our results showed that the extract had similar effect on *Ae. aegypti* strain from Hue in comparison to laboratory *Ae. aegypti* (Figure 4).

In the negative control group, the percentage of larvae died after 3 days was 1.33% (Figure 4A). The concentration of 20 ppm reached maximum effect after 32 hours post-exposure with 8% of larvae were killed. When the concentration increased, to 40 ppm and higher, 90.67% or more of larvae died at day 3. At highest concentration of 1000 ppm, all of larvae was killed within 24 hours post-exposure. LC50 of *S. nigrum* extract on Hue strain of *Ae. aegypti* was 25.07 ppm (Figure 4B).

Larvicidal activity of S. nigrum extract on Ae. albopictus, strains of Hue and Dak Lak

Larvicidal activity of *S. nigrum* extract on *Ae. albopictus* strains collected from Hue (Figure 5) and Dak Lak (Figure 6) was tested.

The results showed that the lowest extract concentration of 20 ppm had no toxicity to larvae in comparison to the control groups. However, just above the lowest concentration, the extract had very strong larvicidal activity. At day 3, extract at 40 ppm killed 76% of Hue (Figure 5A) and 78.67% of Dak Lak (Figure 6A) strain larvae. The concentrations of 200 ppm or higher killed 100% of larvae at day 3. The highest concentration of 1000 ppm killed all of larvae just within 8 hours post-exposure. LC50 of the extract on Hue and Dak Lak strains of *Ae. albopictus* were 32.27 and 33.35 ppm, respectively (Figures 5B and 6B).

Discussion

DHF outbreaks are always a health problem in Vietnam with severe outcome and cost to the country (Lee *et al*., 2017). The most important and effect methods to prevent the disease are vector control approaches (WHO, 2003). The target of vector control is to interrupt

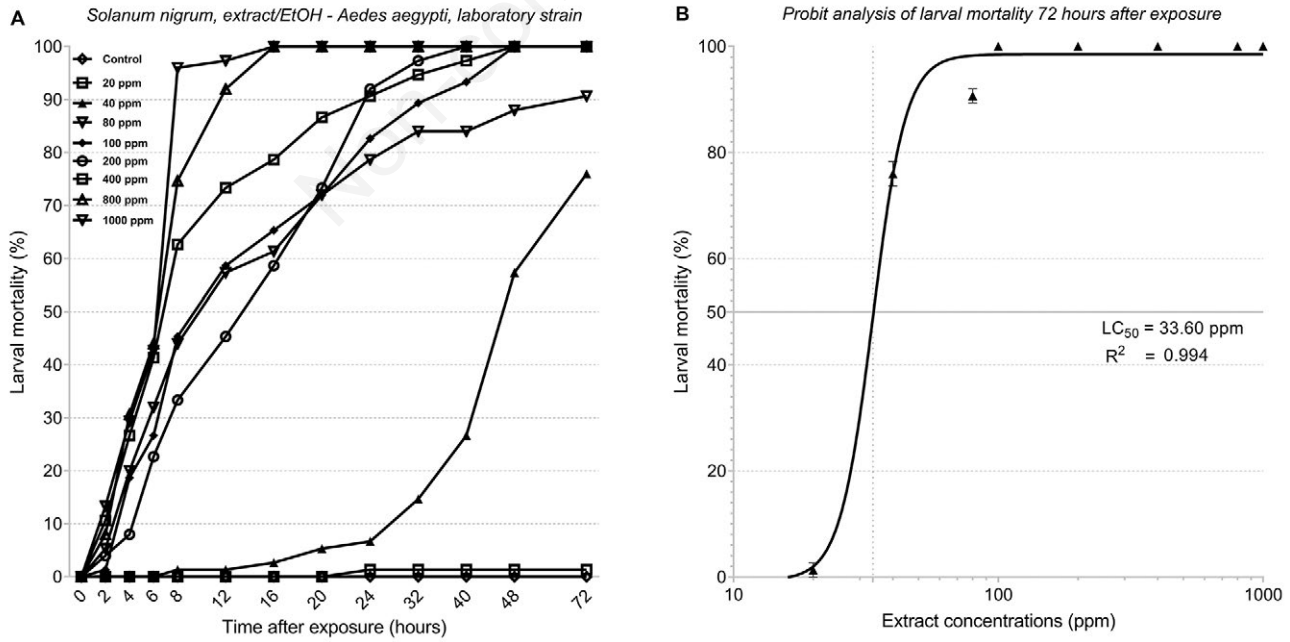

Figure 3. Larvicidal activity of *S. nigrum* ethanolic extract on *Ae. aegypti,* laboratory strain. *S. nigrum* was extracted in absolute ethanol. Larvae were exposed to 20 – 1000 ppm extracts for 1 hour and followed up for 72 hours. (A) Numbers of dead larvae at different time points were recorded. (B) LC50 determination by probit analysis of larval mortality 72 hours after exposure. Each bioassay was repreated 3 times.

humans and mosquitoes interaction. Many studies focused on plant products to avoid side effects of chemicals. Several researches tried with essential oils to discover their topical repellency (Trongtokit *et al*., 2005, Soonwera and Phasomkusolsil, 2016). Some scientists tested ovicidal and adulticidal properties of plants with very promising results, such as *Asparagus racemosus* (Govindarajan and Sivakumar, 2014b) or *Erythrina indica* (Govindarajan and Sivakumar, 2014a)... Normally, the search for plant products was based on the trials with 3 common mosquito families *Aedes, Culex* and *Anopheles* (Phasomkusolsil and Soonwera, 2010, Phasomkusolsil and Soonwera, 2011). Some studies focused on *Aedes* only (de Omena *et al*., 2007, Silva *et al*., 2006). The focus on *Aedes* mosquito in vector control research means that this family is very important subject, particularly *Ae. aegypti*.

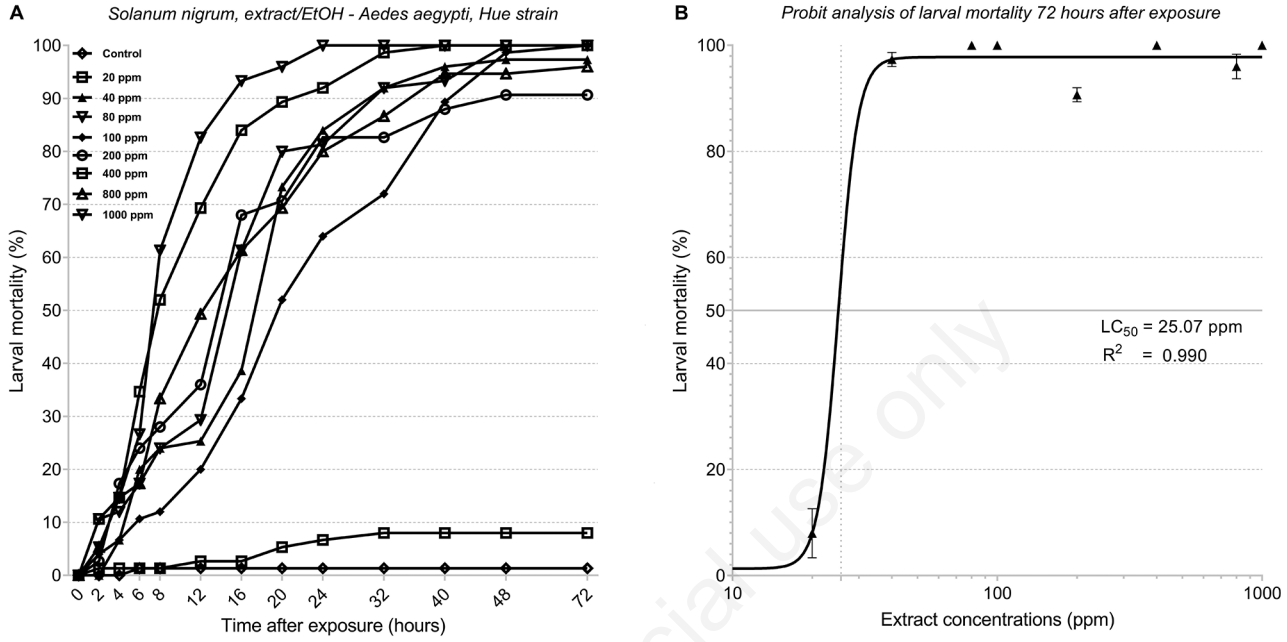

Figure 4. Larvicidal activity of *S. nigrum* ethanolic extract on *Ae. aegypti*, Hue strain. Larvae were exposed to 20 – 1000 ppm extracts for 1 hour and followed up for 72 hours. (A) Numbers of dead larvae at different time points were recorded. (B) LC50 determination by probit analysis of larval mortality 72 hours after exposure. Each bioassay was repeated 3 times.

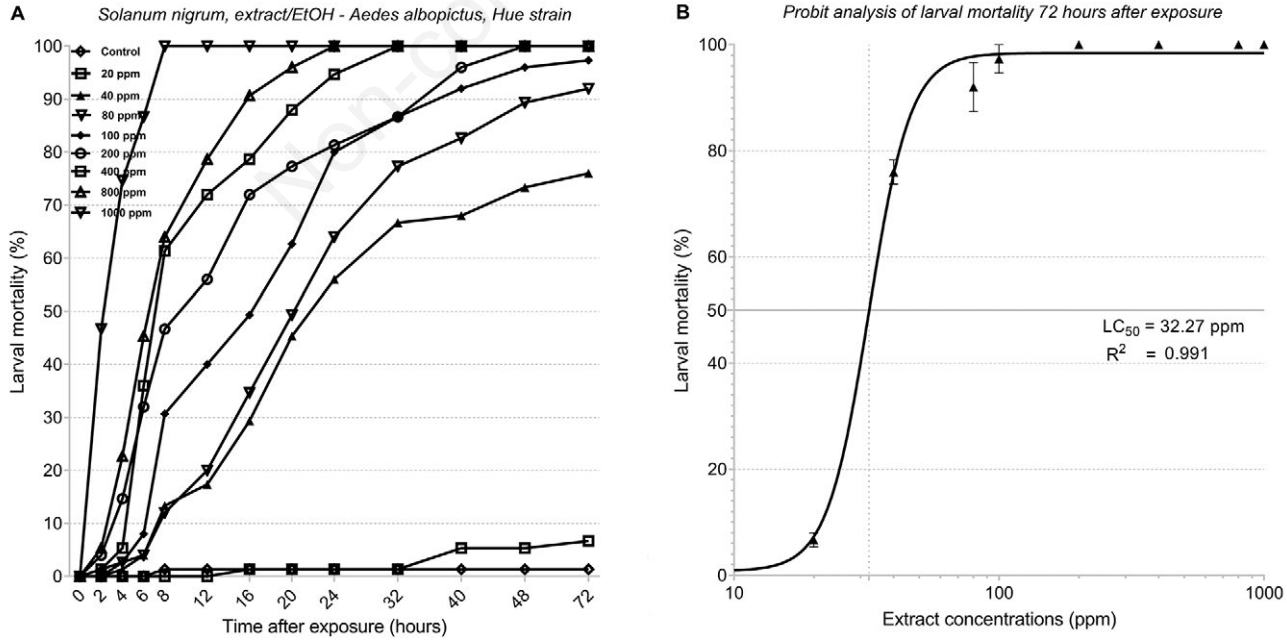

Figure 5. Larvicidal activity of *S. nigrum* ethanolic extract on *Ae. albopictus*, Hue strain. Larvae were exposed to 20 – 1000 ppm extracts for 1 hour and followed up for 72 hours. (A) Numbers of dead larvae at different time points were recorded. (B) LC50 determination by probit analysis of larval mortality 72 hours after exposure. Each bioassay was repeated 3 times.

Herein this study, we showed for the first time the larvicidal activity of a plant on *Aedes* mosquitoes in Vietnam. The plant we chose was *S. nigrum*, a common plant in countryside locations in Northern Vietnam. The initiative of our study was to seek plant(s) with larvicidal application for mosquito control, focusing on *Ae. aegypti* and *Ae. albopictus*.

Although *S. nigrum* has been investigated the larvicidal activity on some common medical mosquitoes, such as *Culex* and *Aedes*, (Patel *et al*., 2018, Rawani *et al*., 2010), it is necessary to study if this quality exists in the plants growing in Vietnam since a plant species may express different phenotypes in different soils. For example, the content of antimalarial active compound namely artemisinin in *Artemisia annua* grown in Sichuan was higher than the plants grown in Beijing (Tu, 2011).

We started with simple method of plant processing which could be done by any people, so that they can apply the method at home reasonable time and cost. The simple method we tried was the preparation of plant infusion. Our data showed that infusion of *S. nigrum* killed laboratory *Ae. aegypti* larvae. However, the larvicidal activity occurred in very high infusion concentrations (10% and 20% concentrations). Highly concentrated infusion could be impractical ways to mosquito control with larvicidal approaches. Although the infusion showed very limited potential in mosquito control practice since it required very high concentration to get larvitical activity, it suggested that *S. nigrum* contained components with desired bioactivities. These components from plants could be extracted with appropriate solvent. The commonest solvent is ethanol which could be used to extract many plants (de Omena *et al*., 2007, McKern and Parnell, 1964, Govindarajan and Sivakumar, 2014a).

In addition to the bioassays with infusion, we tried to produce plant extract with ethanol. Ethanol was the solvent of choice amongst many available solvents such as hexane, ether, chloroform… because its use was approved in reality (Grzybowski *et al*., 2013, Edwin *et al*., 2013). Our data showed that ethanolic extract of *S. nigrum* had larvicidal activity in both laboratory and wild mosquitoes. The laboratory *Ae. aegypti* was used to screen the bioactivity of the plant because this strain is native and is a referent model to study insecticides and repellents. In comparison to infusion, ethanolic extract of *S. nigrum* expressed very strong activity. The larvicidal activity occurred in water containing the extract at concentration as low as 40 ppm. From the extract concentration of 100 ppm or more, all of larvae was killed in bioassays with LC50 of 33.6 ppm. This suggests that the extract could be used to eliminate larvae.

Beside bioassays with laboratory mosquitoes, we also carried out tests with two species *Ae. aegypti* and *Ae. albopictus* derived from different locations. This is necessary steps because wild mosquitoes might have insecticide resistance that lacks in laboratory strains. Interestingly, our data revealed that ethanolic extract of *S. nigrum* had larvicidal properties on both *Aedes* species from different geographic locations in Vietnam. The wild mosquitoes came from two distant provinces to make sure that they are isolated and could be genetically different. This results suggested that the effect of ethanolic extract of *S. nigrum* is not depend on geographic origins of *Aedes* mosquitoes, meaning that it could be effective to the mosquitoes from different provinces in Vietnam.

When a product is proposed for applications, the most important aspect is the toxicity of the product. In case of *S. nigrum,* the question is the safety of the plant infusion and extract for humans and pets. In fact, this plant has been used as a herbal medicine as well as food in Vietnam for long time. Usage of this plant was stated in a book namely "Medical plants and remedies in Vietnam" (Loi, 1977). According to the instruction, trunks, branches and leaves of *S. nigrum* can be cooked and served as vegetable. Wounded or burn skin can be cured with extract from fresh plant. However, the berries of this plant could not used as food nor for treatment because of toxins.

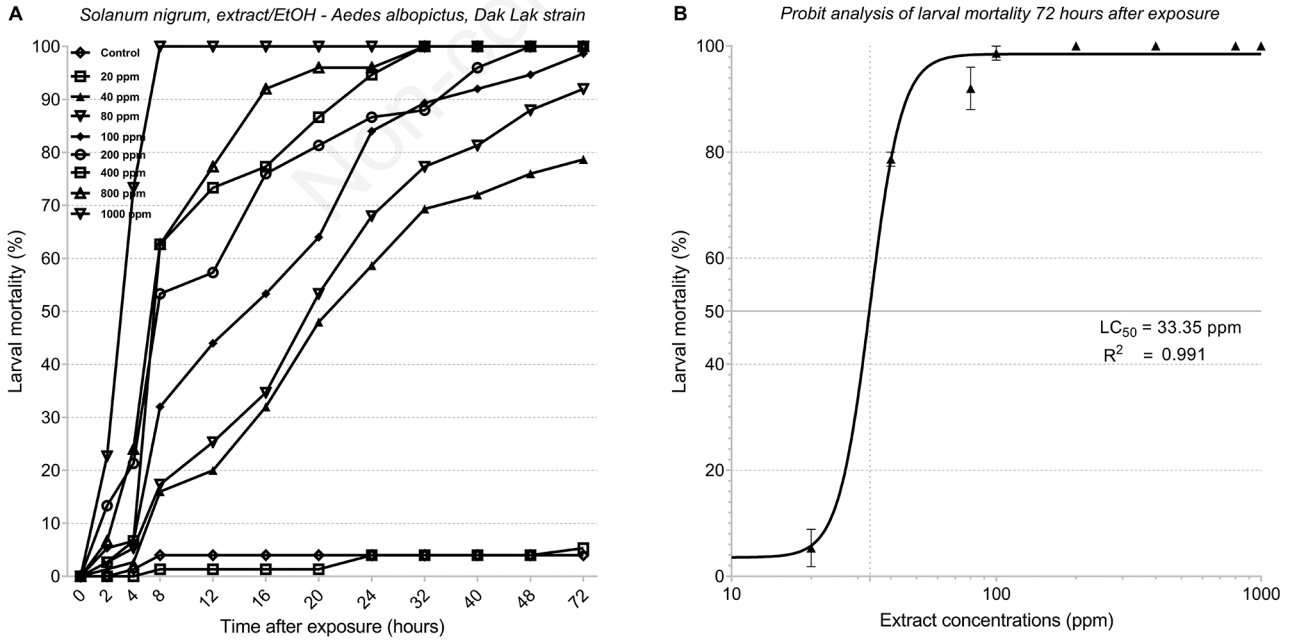

Figure 6. Larvicidal activity of *S. nigrum* ethanolic extract on *Ae. albopictus,* Dak Lak strain. Larvae were exposed to 20 – 1000 ppm extracts for 1 hour and followed up for 72 hours. (A) Numbers of dead larvae at different time points were recorded. (B) LC50 determination by probit analysis of larval mortality 72 hours after exposure. Each bioassay was repreated 3 times.

Conclusions

Our study showed that infusion and extract *S. nigrum* carry larvicidal activity to laboratory and wild *Ae. aegypti and Ae. albopictus*. The infusion could kill all of tested larvae at very high concentration. Ethanolic extract showed strong larvicidal effect at concentration as low as 40 ppm, and reached maximum effects at concentration of 800 ppm. LC50 values on tested mosquito strains were between 25.05-33.60 ppm. The data suggest that the plant extract may be used as control measure against *Aedes* larvae.

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