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Phytochemical screening of *Simarouba amara* seed oil: A potent mosquitocidal effect against malarial vector, *Anopheles stephensi*

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Abstract
Humanity has employed medicinal plants as a source of medications for many thousands of years. The evergreen flowering tree plant *Simarouba amara* is also known as bitterwood, dysentery barks, Laxmi Taru, and paradise tree. It uses its seeds to make edible oils. Mosquitoes spread a wide range of illnesses like malaria, yellow fever, dengue, filariasis, and chikungunya in addition to the terrible biting nuisance. The effectiveness of plant extracts against mosquito larvae has been the subject of numerous studies worldwide. The goal of the current study was to evaluate the phytochemical composition, larval and pupal toxicity, ovicidal activity, adulticidal activity, and repellant efficacy of *Simarouba amara* oil against the malaria vector *Anopheles stephensi*. Phytochemical analysis of *S. amara* oil revealed the presence of alkaloids, flavonoids, phenols, tannins, proteins, carbohydrates, steroids, Triterpenoids. GC/MS analysis identified at least 7major bioactive compounds in the *S. amara* oil. In mosquitocidal bio-assay, LC_{50} of *S. amara* oil against *An. stephensi* larvae and pupae were 231.185 ppm (larva I), 267.066 ppm (II), 301.830 ppm (III), 356.892 ppm (IV), and 435.970 ppm (pupa). In ovicidal assay, egg hatchability was reduced by 100% after treatment with 400 ppm of *S. amara oil*. In adulticidal assays the LC_{50} values were 332.375ppm for 50% of adult mortality of An. stephensi, and repellant efficiency of *S. amara* oil against *An. stephensi* were repellant by 64% after the treatment of 100ppm. Overall, our results highlighted that *S. amara* oil could be useful candidates to develop bio-formulated mosquitocidal effective against malarial vector, *An. stephensi*.

Keywords: *An. stephensi*, malaria, oil formulations, phytochemicals, mosquito repellant

Introduction
Mosquitoes are significant carriers of virulent infections and bloodsuckers that could spread globally as pandemics or epidemics among the expanding human and animal populations. Contagious disease emergence and reemergence is a serious global public health concern. One of the most significant carriers of malaria in India and other West Asian nations is the mosquito *Anopheles stephensi*. Currently, insecticide-treated nets (ITNs) and indoor residual spraying treatments (IRS) are the main methods used to control malaria vectors. However, these methods come with a number of drawbacks, such as the emergence of pesticide-resistant strains, risks to human health, and non-target organisms [1, 2]. In order to create efficient and dependable Integrated Vector Management (IVM) strategies, the creation of unique and environmentally friendly mosquito control techniques is a crucial requirement [3].
Phytochemicals are a powerful group of chemicals that are derived from natural resource, especially with plants origin. Phytochemicals are complex organic molecules that are found in plants. So they are known as therapeutic constituents [4]. Scientist's focuses their interest mainly on phytochemicals and use of phytochemicals is increasing frequently more because of the harmful side effects of the synthetic compounds on human health as well as on environmental and non-target organisms [5]. Secondary metabolites such as phenolics, flavonoids, terpenoids, coumarins and alkaloids are active against some of the target insects and potentially suitable for integrated pest management (IPM) programs, as they could lead to the development of new classes of safer insect control agents [6]. Plant-borne chemicals can act as an ovicides, larvicides, pupicides, adult repellents and oviposition deterrents against a wide number mosquito species of economic importance [7]. Recently, mosquito control using phytochemical products arising as a valuable alternative, due to their reduced toxicity towards non-targets and high biodegradability [8]. Simarouba are monospecific members of the Simaroubaceae family that have been widely documented in several pharmacopoeias for their high medicinal value [9]. *Simarouba amara* (synonym *Simarouba glauca*) is a paradise tree or laxmitaru in India, and it has a high medicinal value. Glaucarubinone, a natural product of Simarouba, is an antimalarial drug [10] and has been shown to have anticancer activity in pancreatic cancer via downregulation of P21-activated kinases (PAK) [11]. We investigated the preliminary phytochemical analysis of *S. amara* oil and evaluated the bioactive compounds using gas chromatography-mass spectrometry in this study (GC-MS). Furthermore, the larval/pupal toxicity, adulticidal, repellant, and ovicidal activity of *S. amara* seed oil against the malarial vector, *An. stephensi*, were investigated.

Materials and methods

Collection and Preparation of oil

Fresh *S. amara* seeds were collected from the selected trees grown in Coimbatore, Erode, Krishnagiri and Dharmapuri districts, Tamil Nadu, India. Collected seeds were depulped, processed, air dried, packed and stored for further analysis. *S. amara* seed oil has been extracted by soxhlet apparatus using organic solvents viz., petroleum ether and n-Hexane. Dried seeds were weighed and macerated with blender and the powder was subjected to extraction of oil. About 100gms of *S. amara* seed powder was filled in thimble (Whatmann No: 1 filter paper) and placed inside the butt tube of soxhlet apparatus. About 350 ml solvent was taken in the flat bottom flask and 2 pieces of porcelain bits were added to avoid bumping. Fit the butt tube with the condenser and to the flask. Proper water circulation was provided to aid condensation of solvent vapours. The heating mantle was set at 60^{o} C (with respect to boiling point of solvent), the solvent gets vaporized slowly and enters the butt tube. Due to the cooling system (condenser) above the butt tube, these vapours get condensed and collected in the butt tube after a threshold or saturation level, the condensed solvents overflow and collected into the flat bottom flask along with oil. After distillation, oil had been recovered from the solvent by subjecting to rotary evaporator.

Phytochemical screening of *S. amara* seed oil

Harborne [12], Trease and Evans [13], and Sofowara [14] used standard procedures to detect the presence of active biomolecules such as Alkaloids, Saponins, Flavonoids, Steroids, Terpenoids, Tannins, Phenols, Carbohydrates, and Proteins in *S. amara* seed oil.

Gas chromatography-mass spectrometry (GC–MS) analysis

The Perkin-Elmer Clarus 680 system (Perkin-Elmer, Inc., USA) was used for GC-MS analyses of *S. amara* seed oil, which was equipped with a fused silica column packed with Elite-5MS) capillary column (30 m in length×250 µm in diameter×0.25 µm in thickness). The carrier gas was pure helium gas (99.99 %) at a constant flow rate of 1 mL/min. An electron ionisation energy method with a high ionisation energy of 70 eV (electron Volts), 0.2 s scan time, and fragments ranging from 40 to 600 m/z was used for GC-MS spectral detection. The injection volume was 1 L (split ratio 10:1), and the injector temperature was kept at 250 °C (constant). The column oven temperature was set at 50 °C for 3 minutes, then increased at 10 °C per minute up to 280 °C before being increased to 300 °C for 10 minutes. The phytochemical content of the test samples was determined by comparing their retention time (min), peak area, peak height, and mass spectral patterns to those in the spectral database.

An. stephensi mosquito rearing

The National Centre for Disease Control (NCDC) Mettupalayam field station provided *An. stephensi* eggs (Tamil Nadu, India). For hatching, eggs were transferred to laboratory conditions [27 2 °C, 75-85 percent R.H., 14:10(L/D) photoperiod] and placed in 18 13 4-cm plastic containers containing 500 ml of tap water. Larvae were fed daily a 3:1 (w/w) mixture of dog biscuits (Pedigree, USA) and hydrolyzed yeast (Sigma-Aldrich, Germany). Larvae and pupae were collected, transferred to 500 mL glass beakers of dechlorinated water, and tested in subsequent experiments.

Larvicidal and pupicidal toxicity in laboratory conditions

Twenty-five *An. stephensi* larvae (I, II, III, or IV instars) or pupae were placed for 24 hours in a glass beaker filled with 250 ml of dechlorinated water plus the desired concentration of *S. amara* oil (100, 200, 300, 400, and 500 ppm). Each concentration tested received 0.5 mg of larval food. Each concentration was tested five times on all instars. Control mosquitos were exposed to the corresponding concentration of the solvent for 24 hours. The percentage mortality was calculated as follows:

Percentage mortality = (number of dead individuals / number of treated individuals) × 100

Ovicidal activity

In ovidal activity assays, *An. stephensi* eggs were collected using ovitraps, which are Petri dishes with a diameter of 60 mm that are lined with filter paper and contain 100 ml of water, as per Su and Mulla [15]. Ovitraps were kept in the cages for two days after the ladies' blood meal. Using a photomicroscope, the eggs placed on the filter paper lining were analysed (Leica ES2, Germany). Then, a cage made of six glass cups was used to hold the eggs (diameter: 6 cm). The following *S. amara* oil doses were added to five of them together with water: 100, 150, 200, 250, and 300 ppm (oil). Distilled water was used in the cup used as a control. In each cup, 100 eggs were put. For each dosage, five replicates were performed. Following treatment, after counting the eggs under a microscope, the eggs from each concentration were transferred to cups of distilled water for hatching evaluation. Based on the inability of eggs with closed operculae to hatch, the percentage of egg mortality was estimated. 48 hours after treatment, the hatch rates were calculated using the following formula:

Egg mortality % = (number of hatched larvae / total number of eggs) × 100

Adulticidal bioassay

We employed adult female mosquitoes that had been fed sugar (5–6 days old). In order to create varied concentrations, *S. amara* oil was diluted with acetone. Filter sheets (140 mm x 120 mm) were impregnated with the diluted oil. As a control, blank paper made only of ethanol was used. Overnight, the papers were allowed to dry at room temperature so that the ethanol may evaporate. Prior to testing, freshly made impregnated papers were used. The bioassay was carried out using the WHO technique in an experimental set made up of two 12544 mm cylindrical plastic tubes (1981). A tube was utilized to contain the mosquitoes before and after the exposure times, and another tube was used to expose the mosquitoes to the plant extract. The papers that had been impregnated were rolled up and put in the exposure tube. Each tube has a wire screen with a mesh size of 16 to shut one end. Twenty sucrose-fed and blood-starved mosquitoes were placed into the tube, and the extracts' mortality effects were tracked every 10 minutes for three hours of exposure. The mosquitoes were put in the holding tube at the conclusion of exposure times of 1, 2, and 3 hours. During the 24-hour holding period, cotton pads soaked in a 10 percent sugar solution containing vitamin B complex were put in the tube. After 24 hours, the mosquitoes' mortality was noted. The aforementioned technique was completed three times for each concentration of oil.

Adult Repellency activity

The cotton pads were stored in glass containers after being soaked in *S. amara* oil at various concentrations together with goat blood. Each container contained one treatment pad and one control pad that were positioned in opposite directions. Each container contained twenty adult mosquitoes. On the treated and control pads, the number of mosquitoes landing was counted. The following formula was used to determine the percentage protection provided by *S. amara* oil and control:

$(C-T/C) \times 100$. Where C–The number of mosquitoes on control, T – The number of mosquitoes on treated.

Data analysis

For all analyses, the SPSS software package 16.0 version was utilized. Data on mosquito toxicity from laboratory assays were converted into arcsine/ proportion values, and a two-way ANOVA with two factors was used to examine the results (i.e., dosage and mosquito instar). Using the Tukey's HSD test, means were separated. Additionally, using the Finney method [16] to calculate LC_{50} and LC_{90}, probit analysis was used to assess mosquito mortality data from lab experiments. Ovicidal data were converted into arcsine √proportion values and subjected to a two-factor ANOVA analysis (i.e. dose and species). Using the Tukey's HSD test, means were separated ($P < 0.05$).

Results and Discussion

Preliminary phytochemical analysis of *S. amara* oil

Various chemical experiments were performed on the *S. amara* seed oil to find secondary plant metabolites. The findings showed that the *S. amara* seed oil contains components that are medicinally active, including alkaloids, flavonoids, phenols, tannins, steroids, and triterpenoids. (Table 1 & Figure 1). Similarly Pawar *et al* [17]. reported the quantitative estimation of bioactive components from *Simarouba glauca* leaves extract. Phytochemicals were screened for Alkaloids, Carbohydrates, Glycosides, Coumarins, Proteins & Amino acids, Phenolic compounds, Tannins, Flavonoids, and Terpenoids.

Table 1: The primary phytochemicals screening of *Simarouba amara* seed oil

Phytochemical	Petroleum ether
Phenolic	+
Tannins	+
Flavonoids	++
Alkaloids	+
Steroids	+
Triterpenoids	++

+ = Present and - = Absent

GC-MS analysis of *S. amara* oil

Seven peaks were found in the GC-MS chromatogram of *S. amara* oil, and they were identified as the bioactive chemicals by comparing their peak retention times, various peak areas (%), and mass spectral fragmentation patterns (Figure 1). *S. amara* oil results revealed 7 chemicals (Table 2). Similar to this, GC-MS analysis of *C. sinensis* seed oil reveals that it contains 100% of linoleic acid, 33.55 % of palmitic acid, 21.96 % of stearic acid, 21.72 % of caffeine, and 14.49 % of vitamin E [18]. According to pharmacokinetic tests, all of the participants in the fasting group had greater plasma concentrations. Additionally, it was discovered that the oil from *Raphanus sativus* seeds is rich in substances like oleic acid and erucic acid, which account for 30.011%, 5.464%, and 16.411% of the total oil, respectively [19].

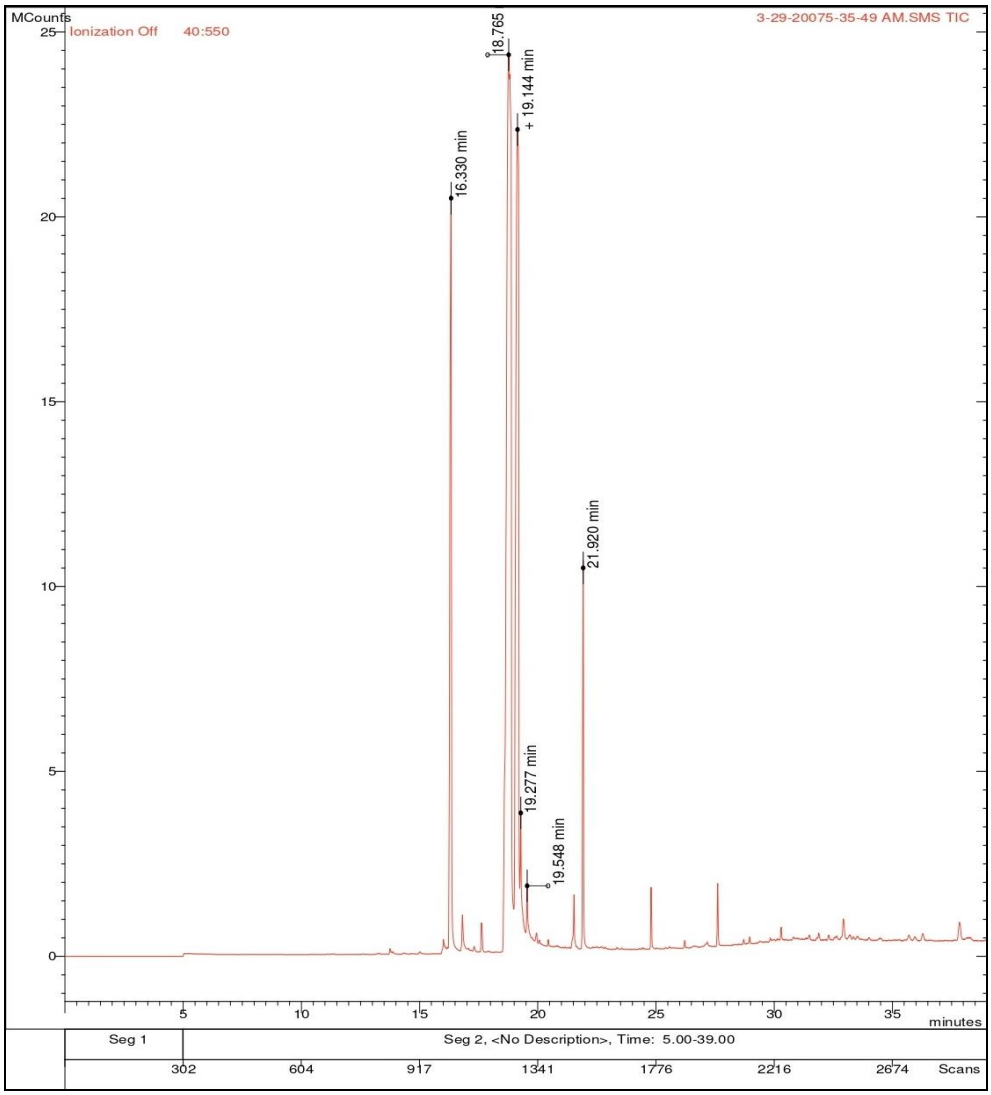

Fig 1: GCMS spectrum of *S. amara* seed oil

Table 2: GCMS analyses of *S. amara* oil

RT (Min)	Compound	Molecular Formula	Molecular weight
16.330	palmitic acid	$C_{16}H_{32}O_2$	256.43 g/mol
18.765	Oleic acid	$C_{18}H_{34}O_2$	282.468 g/mol
18.786	Stearic acid	$C_{18}H_{36}O_2$	284.484 g/mol
19.144	Eicosenoic Acid	$C_{21}H_{40}O_2$	324.549 g/mol
19.277	Arachidic acid	$C_{20}H_{40}O_2$	312.538 g/mol
19.548	Behenic acid	$C_{22}H_{44}O_2$	340.592 g/mol
21.920	Lignoceric acid	$C_{24}H_{48}O_2$	368.646 g/mol

Larval/pupal toxicity of *S. amara* oil against *An. stephensi*

The *S. amara* oil shown toxicity against *An. stephensi* larvae and pupae in experimental settings. The impact was dose-dependent, which is consistent with earlier findings on other plant extracts [20, 21]. *S. amara* oil has LC50 (LC90) values of 231.185 (475.939) for larva I, 267.066 (544.814) for larva II, 301.830 (592.844) for larva III, 356.892 (390.340) for larva IV, and 435.970 (825.894) for pupa (Table 3). The presence of the botanical chemicals discovered by GC/MS analysis, which primarily influence the midgut epithelium and subsequently affect the gastric ceaca and the malpighian tubules in mosquito larvae, may be related to the larvicidal activity of the *S. amara* oil [22].

Table 3: Larval and pupal toxicity effect of *S. amara* seed oil against malarial vector *An. Stephensi*

Mosquito life stages	LC_{50} (LC_{90}) (ppm)	95% confidence Limit		Regression equation	χ^2 (*df*=4)
		LC_{50} (LC_{90})			
		LCL	UCL		
1st Instar	231.185 (475.939)	204.561 (438.113)	254.882 (527.461)	y= -1.211+1.345 x	1.345*n.s.*

2nd Instar	267.066 (544.814)	239.586 (497.189)	292.803 (612.199)	y = -1.23 +0.989 x	0.989 *n.s.*
3rd Instar	301.830 (592.844)	274.688 (538.305)	329.198 (671.554)	y = - 1.329+0.004x	1.896 *n.s.*
4th Instar	356.892 (390.340)	327.745 (604.167)	390.340 (776.177)	y = - 1.447+0.004x	2.410 *n.s.*
Pupa	435.970 (825.894)	395.087 (716.110)	493.925 (1010.282)	y = -1.433 +0.003x	0.794 *n.s.*

Mortality rates are means ± SD of five replicates, No mortality was observed in the control
Within each row, means followed by the same letter(s) are not significantly different (P<0.05),
LC_{50}=lethal concentration that kills 50% of the exposed organisms,
LC_{90}=lethal concentration that kills 90 % of the exposed organisms,
χ^2 = *chi*-square value,
n.s. = not significant (α=0.05)

Ovicidal activity

In ovicidal investigations, *S. amara* oil treatment reduced the hatchability of *An. stephensi* eggs, which had a 100% mortality rate after 400 ppm treatment, in contrast to control eggs, which had a 100% hatchability rate (Table 4). The majority of Culex egg rafts treated to asafoetida (55.8 %) or garlic (73.1 %) essential oils failed to hatch, and the larvae of the few that did typically perished in their first instars, according to Muturi *et al* [23]. After being exposed to *Cymbopogan citratus* essential oil, 300 ppm of *Culex quinquefasciatus* displayed 100% ovicidal activity [24].

Table 4: Ovicidal activity of *S. amara* oil against malarial vector, *An. stephensi*

Treatment	**Percentage of egg hatchability**					
	Concentrations (ppm)					
***S. amara* seed oil**	**Control**	**100**	**200**	**300**	**400**	**500**
	97.5±0.50	59.34±0.78	46.2±1.13	19.36±0.68	NH	NH

Values were means ± SD of five replicates. Different letters indicated significant differences (P<0.05**)**
NH-No hatchability (100% mortality)

Adulticidal activity

The adulticidal action of *S. amara* oil against *An. stephensi* adults is shown in Table 5. The adult died after exhibiting agitated behavior and abnormal wagging at increasing intensities. According to the concentration, the fatality rates were commensurate. The LC_{50} and LC_{90} values for *S. amara* oil were 332.375 and 717.024 ppm, respectively. The effectiveness of various solvent extracts of *Ocimum caninum*, *Ocimum gratissimum*, *Chromolaena odorata*, and *Datura stramonium* against adult *Anopheles gambiae* was previously investigated by Afolabi *et al*. [25]. When tested at LC_{50} (0.82-4.58 mg/l) and LC_{90} (2.38-8.33 mg/l), the extracts exhibited adulticidal action. *Ocimum gratissimum* and *Datura stramonium* had the highest fatality rates, with LC_{50} and LC_{90} values of 2.35 mg/l, 4.75 ml/l, and 0.82 mg/l, 2.38 mg/l, respectively. *Chromolaena odorata* demonstrated the lowest LC_{50} (4.52 mg/l) and LC_{90} (8.32 mg/l) values for adulticidal activity.

Table 5: Adulticidal activity of *S. amara* seed oil against malarial mosquito, *An. Stephensi*

Treatment	**Concentration (ppm)**	**Mortality (%) (mean ± SD)**	**LC_{50} (LCL-UCL)**	**LC_{90}(LCL-UCL)**	**x^2**
S. amara oil	Control	0.0 ± 0.0		717.024	
	100	23.48± 1.37	332.375	(629.025 -859.945)	0.750n.s
	200	29.58 ±0.97	(297.962-370.448)		
	300	47.42 ± 0.77			
	400	59.22 ± 0.68			
	500	70.96 ± 0.66			

Mortality rates are means ± SD of five replicates.
No mortality was observed in the control.
LC_{50} = lethal concentration killing 50 % of the insects
LC_{90} = lethal concentration killing 90 % of the insects
χ 2 = chi-square d.f. = degrees of freedom
n.s. = not significant (α=0.05)

Adult repellent activity

Table 6 shows the ability of the *S. amara* oil to repel adult *An. stephensi*. The test was conducted from sundown till dark. Different extract concentrations were applied by human volunteers, and it was discovered that the repellent protection was only partially realized at the 100% concentration with a protection percentage of 64.88 % and was extremely low at the 20% concentration with a protection percentage of 19.64 %. On the other hand, the following concentration did not result in a notable level of protection. Instead, protection levels of 36.30, 42.85, and 56.54 % were observed in relation to concentrations of 40, 60, and 80 %. From 17 pm to pm, the

repellency was measured after every 30 minutes. The fed mosquitoes were only recorded here and the rest which showed landing activity alone were not taken to the account. Our results compared to previous reports Afolabi *et al*. [25] under laboratory conditions (28 ±2 °C, 75± 5% RH, 12L: 12D photoperiod), the effectiveness of the investigated repellant against mosquitoes was assessed at five concentrations: 1.0, 2.0, 3.0, 4.0, and 5.0 mg/l. At 5 mg/l of the extracts, the results revealed complete protections of 90 min for *Ocimum caninum*, 120 min for *Ocimum gratissimum*, 30 min for *Chromolaena odorata*, and 120 min for *Datura stramonium*.

Table 6: Repellent activity of *S. amara* seed oil against malarial vector *An. stephensi*

Mosquito species	Concentration (ppm)	Time post application of repellent (min)						Total	(%) of Protection
		30	60	90	120	150	180		
Anopheles stephensi	Control	47	37	31	22	18	13	146	--
	20	38	29	26	19	12	11	121	19.64
	40	33	24	18	14	10	8	95	36.30
	60	28	21	19	13	9	6	82	42.85
	80	21	14	15	10	8	5	70	56.54
	100	18	10	11	8	7	5	59	64.88

Conclusion

The chemical compounds employed for the phytochemical compounds in the *S. amara* seed oil were identified in this study by the GC-MS analysis. The *An. stephensi* malarial vector's eggs, larvae/pupae, and adult were particularly susceptible to the *S. amara* seed oil. The findings imply that *S. amara* seed oil might be investigated further for a successful programme to reduce mosquito vectors.

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