SUPPLEMENTARY MATERIAL

Neem cake as a promising larvicide and adulticide against the rural malaria vector *Anopheles culicifacies* (Diptera: Culicidae): a HPTLC fingerprinting approach

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Abstract

Mosquitoes are insects of huge public health importance, since they act as vectors for important pathogens and parasites. Here we focused on the possibility of using the neem cake in the fight against mosquito vectors. The neem cake chemical composition significantly change among producers, as evidenced by our HPTLC analyses of different marketed products. Neem cake extracts were tested to evaluate the ovicidal, larvicidal and adulticidal activity against the rural malaria vector *Anopheles culicifacies*. Ovicidal activity of both types of extracts was statistically significant, and 150 ppm completely inhibited egg hatching. LC₅₀ values was extremely low against fourth instar larvae, ranging from 1.321 ppm (NM1) to 1.818 ppm (NA2). Adulticidal activity was also high, with LC₅₀ ranging from 3.015 ppm (NM1) to 3.637 ppm (NM2). This study pointed out the utility of neem cake as source of eco-friendly mosquitocides in Anopheline vector control programs.

Keywords: Anopheline; arbovirus; eco-friendly insecticides; mosquito vectors; ovicidal activity

EXPERIMENTAL

Neem cake material and extraction process

Neem cake samples from two distinct cultivation sites located in Southern India (Tamil Nadu region, detailed information about growth conditions can be given upon request) was kindly provided by Italian importers. Following the methods reported by Benelli et al. (2014), neem cake (0.5 kg) was extracted with methanol at room temperature twice for 4 days, obtaining, after evaporation of the solvent, 49 g of the total methanol extract dry extract (NM1). The same procedure was repeated on 0.5 Kg of neem cake using ethyl acetate as solvent extraction, obtaining the total ethyl acetate extract (NA1). Similarly, NM2 and NA2 were obtained from the second neem cake product. 1 g of the each sample was separately dissolved in 100 ml of acetone (stock solution) and considered as 1% stock solution. From this stock solution, different concentrations were prepared ranging from 0.5 to 2.5 ppm for larvicidal assays; 1.5 to 7.5 ppm for adulticidal assays, and 50 to 550 ppm for ovicidal assays.

High performance thin layer chromatography

Chromatographic equipment: glass plates 20 cm × 10 cm (Merck, Darmstadt, Germany) with glass-backed layers silica gel 60 (2 µm thickness). Before use, plates were prewashed with

methanol and dried for 3 min at 100 °C. The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of the instrumental sequence: deposition of the sample by Linomat 5 sample applicator using 100 µL syringes and connected to a nitrogen tank; development of the plate in automatic and reproducibly developing chamber ADC 2, containing twin trough chamber 20 × 10 cm and saturated with the same mobile phase, Toluene/Ethyl acetate 4:6 (v/v); derivatization by Immersion device III and TLC Plate Heater III; computer inspection by TLC visualizer linked to winCATS software; registration and documentation of derivatised plates by CAMAG DigiStore2 digital system with winCATS software 1.4.3

Sample application: the dried extracts of the analysed samples were weighted and dissolved in methanol (30 mg/mL). Filtered extracts were applied with nitrogen flow. The operating conditions were: syringe delivery speed, 10 s µL⁻¹ (100 nL s⁻¹); injection volume, 4 µL; band width, 8 mm; distance from bottom, 8 mm.

Development and derivatization: the length of the chromatogram run was 70 mm from the point of application. The developed layers were allowed to dry for 3 min at 100 °C and then derivatised with a selected solution, i.e. anisaldehyde (2 mL *p*-anisaldehyde, 10 mL H₂SO₄, 20 mL AcOH in 170 mL MeOH). Finally, the plates were warmed for 5 min at 120 °C before inspection. All treated plates were then inspected under a UV (Ultra Violet) light at 254 or 366 nm or under white light WRT (reflectance and transmittance), before and after derivatization. Only part of the images of the HPTLC plates, obtained in different conditions, were reported, because of the available space in this report, but the other images are available under request.

Validation: sample solutions of the extracts were found to be stable at 4 °C for at least one month and for at least 3 days on the HPTLC plates. Repeatability was determined by running a minimum of three analyses. RF values for main selected compounds varied ± 0.02%. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on pre-washing of HPTLC plates with methanol.

Anopheles culicifacies rearing

Eggs and larvae of *Anopheles culicifacies* were collected from Department of Rice, AICRIP – Rice Research Centre (Tamil Nadu Agricultural University, Coimbatore, India).

Following the method reported by Murugan et al. (2015), the eggs were transferred to laboratory conditions [$27\pm 2^{\circ}\text{C}$, 75–85 % R.H., 14:10 (L:D) photoperiod] and placed in plastic containers containing 500 ml of tap water, waiting for larval hatching. Larvae were reared in the plastic containers described by Chandramohan et al. (2016), and fed daily with a mixture of crushed dog biscuits (Pedigree, USA) and hydrolysed yeast (Sigma-Aldrich, Germany) at 3:1 ratio (w:w). Water was renewed every 2 days. The breeding medium was checked daily and dead individuals were removed. Breeding containers were kept closed with muslin cloth to prevent contamination by foreign mosquitoes. Larvae and pupae for experiments were collected daily from culture containers and transferred to glass beakers containing 500 ml of water (Dinesh et al., 2015; Chandramohan et al. 2016).

Ovicidal potential

Following the method by Su and Mulla (1998), in ovicidal activity experiments, *An. culicifacies* eggs were collected placing ovitraps (i.e., Petri dishes, diameter 60 mm, lined with filter paper and containing 50 ml of water) inside each cage. All ovitraps were stored in the cages for 2 days from the blood meal of mosquito females. The eggs laid on filter paper lining were examined using a photomicroscope (Leica ES2, Germany). The eggs were placed in a cage with six glass cups (diameter: 6 cm). Five of them were filled with water plus neem cake treatments formulated at the following doses: 50, 150, 250, 350 and 450 ppm. Methanol and ethyl acetate extracts from both neem cake samples were tested. The control cup was filled with distilled water. 100 eggs were placed in each cup. Five replicates were done for each dosage. After treatment, the eggs from each concentration were transferred to distilled water cups for hatching assessment after counting the eggs under microscope. The percent egg mortality was calculated on the basis of non-hatchability of eggs with unopened opercula (Kuppusamy and Kadarkarai, 2008). The hatch rates were assessed 48 h post-treatment using the following formula (Govindarajan et al., 2011):

$$\text{Egg mortality (\%)} = (\text{number of hatched larvae} / \text{total number of eggs}) * 100$$

Larvicidal potential

A laboratory-reared pathogen-free colony of *An. culicifacies* larvae was used for the larvicidal activity. Twenty-five individuals of fourth instars larvae were kept in a 500 ml glass beaker containing 249 ml of dechlorinated water and 1 ml of acetone plus the neem cake desired concentration (0.5, 1, 1.5, 2 and 2.5 ppm, NM1, NA1, NM2, NA2, respectively, were added separately. Larval food was given for the test larvae. At each tested concentration, two to five trials were made and each trial consists of five replicates. The control was 1 ml of acetone plus 249 ml of dechlorinated water. The control mortalities were corrected by using Abbott's formula (Abbott, 1925).

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Control mortality}} \times 100$$

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

Adulticidal potential

Adulticidal bioassay was performed following the WHO method (WHO, 1981). Neem cake extracts were tested at 1.5, 3.0, 4.5, 6.0 and 7.5 ppm, by application on Whatman n. 1 filter paper (size 12×15 cm) lining a glass holding tube (diameter 30 mm; length 60 mm). Control filter paper was treated with the same volume of distilled water. In each test, 20 *An. culicifacies* females were gently transferred into another glass holding tube. The mosquitoes were allowed to acclimatize in the tube for 1 h and then exposed to test tube lined with treated or control paper for 1 h. At the end of exposure period, the mosquitoes were transferred back to the original holding tube, kept for a 24 h recovery period, then mortality was recorded. A pad of cotton soaked with 10% (w:w) glucose solution was placed on the mesh screen at the top of the holding tube.

Data analysis

Following the method by Madhiyazhagan et al. (2015), the ovicidal data were analysed using a general linear model with three factors, the tested neem cake sample, the extraction solvent and the dose) followed by Tukey's HSD test ($P < 0.05$). Larval and adult mortality data

were subjected to probit analysis by Finney (1971). SPSS version 16.0 was used to calculate LC₅₀ and LC₉₀. Results with $P < 0.05$ were considered to be statistically significant.

References

- Abbott WS (1925). A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18:265–266.
- Benelli G, Conti B, Garreffa R, Nicoletti M (2014). Shedding light on bioactivity of botanical by-products: neem cake compounds deter oviposition of the arbovirus vector *Aedes albopictus* (Diptera: Culicidae) in the field. Parasitol. Res. 113:933–940.
- Chandramohan B, Murugan K, Madhiyazhagan P, Kovendan K, Mahesh Kumar P, Panneerselvam C, Dinesh D, Subramaniam J, Rajaganesh R, Nicoletti M, Canale A, Benelli G (2016) Neem by-products in the fight against mosquito-borne diseases: Biototoxicity of neem cake fractions towards the rural malaria vector *Anopheles culicifacies* (Diptera: Culicidae). Asia-Pacif J Trop. Biomed doi: 10.1016/j.apjtb.2015.11.013
- Dinesh D, Murugan K, Madhiyazhagan P, Panneerselvam C, Nicoletti M, Jiang W, Benelli G, Chandramohan B, Suresh U (2015). Mosquitocidal and antibacterial activity of green-synthesized silver nanoparticles from *Aloe vera* extracts: towards an effective tool against the malaria vector *Anopheles stephensi*? Parasitol. Res. 114:1519–1529.
- Finney DJ (1971). Probit analysis. Cambridge University, London. p 68–78.
- Govindarajan M, Mathivanan T, Elumalai K, Krishnappa K, Anandan A (2011). Mosquito larvicidal, ovicidal, and repellent properties of botanical extracts against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). Parasitol. Res. 109:353–367.
- Kuppusamy C, Murugan K (2008). Oviposition deterrent, ovicidal and gravid mortality effects of ethanolic extract of *Andrographis paniculata* Nees against the malarial vector *Anopheles stephensi* Liston (Diptera: Culicidae). Entomol. Res. 38:119–125.
- Madhiyazhagan P, Murugan K, Naresh Kumar A, Nataraj T, Dinesh D, Panneerselvam C, Subramaniam J, Mahesh Kumar P, Suresh U, Roni M, Nicoletti M, Alarfaj AA, Higuchi A, Munusamy MA, Benelli G (2015). *Sargassum muticum*-synthesized silver

nanoparticles: an effective control tool against mosquito vectors and bacterial pathogens.
Parasitol. Res. 114:4305-4317.

Su T, Mulla MS (1998) Ovicidal activity of neem products (azadirachtin) against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae). J. Am. Mosq. Control Assoc. 14:204–209.

WHO (1981) Instructions for determining the susceptibility or resistance of adult mosquitoes to organochlorine, organophosphate and carbamate insecticides: diagnostic test.
WHO/VBC/81–807. WHO, Geneva.

Table S1. Ovicidal potential of neem cake extracts against the rural malaria vector *Anopheles culicifacies*.

Treatment	Egg hatchability (%)						
	Control	50 ppm	150 ppm	250 ppm	350 ppm	450 ppm	550 ppm
NM1	100±0.0 ^a	62.3±1.4 b	48.4±1.1 c	25.6±1.0 d	NH	NH	NH
NA1	100±0.0 ^a	66.3±1.6 b	52.9±1.3 c	28.5±1.6 d	NH	NH	NH
NM2	100±0.0 ^a	69.4±1.2 b	54.2±1.9 c	34.3±1.8 d	17.5±2.5 e	NH	NH
NA2	100±0.0 ^a	76.5±1.0 b	59.5±1.7 c	40.7±1.4 d	NH	NH	NH

NH = no hatchability

Values were means±SD of five replicates

Different letters indicated significant differences (general linear model, Tukey's HSD, P<0.05)

NM1 = neem cake methanol extract, commercial sample 1

NA1 = neem cake ethyl acetate extract, commercial sample 1

NM2 = neem cake methanol extract, commercial sample 2

NA2 = neem cake ethyl acetate extract, commercial sample 2

Table S2. Acute toxicity of different neem cake extracts against fourth instar larvae of the rural malaria vector *Anopheles culicifacies*.

Treatments	LC ₅₀ (LC ₉₀)	95% Confidence Limit LC ₅₀ (LC ₉₀)		Regression equation	χ^2 (d.f.=4)
		Lower	Upper		
NM1	1.321 (3.517)	1.100 (3.037)	1.516 (4.349)	y = 0.771 + 0.584x	0.202 <i>n.s.</i>
NA1	1.504 (4.031)	1.271 (3.388)	1.739 (5.246)	y = 0.763 + 0.507x	0.490 <i>n.s.</i>
NM2	1.531 (3.967)	1.309 (3.356)	1.760 (5.096)	y = 0.806 + 0.526x	0.355 <i>n.s.</i>
NA2	1.818 (4.773)	1.559 (3.867)	2.172 (6.711)	y = 0.789 + 0.434x	0.183 <i>n.s.</i>

LC₅₀ =

lethal concentration that kills 50 % of the exposed organisms

LC₉₀ = lethal concentration that kills 90 % of the exposed organisms

χ^2 = chi-square value

d.f. = degrees of freedom

n.s. = not significant ($\alpha=0.05$)

NM1 = neem cake methanol extract, commercial sample 1

NA1 = neem cake ethyl acetate extract, commercial sample 1

NM2 = neem cake methanol extract, commercial sample 2

NA2 = neem cake ethyl acetate extract, commercial sample 2

Table S3. Adulticidal toxicity of different extracts of neem cake against the malaria vector *Anopheles culicifacies*.

Treatment	LC ₅₀ (ppm) (LCL – UCL)	LC ₉₀ (ppm) (LCL – UCL)	Regression equation	χ^2 (d.f.=4)
NM1	3.015 (1.452-3.967)	6.481 (5.290-9.501)	y=1.115+0.370x	8.808 <i>n.s</i>
NA1	2.954 (0.743-4.088)	6.666 (5.276-11.114)	y=1.020+0.345x	11.126 <i>n.s</i>
NM2	3.637 (3.141-4.070)	8.334 (7.521-9.532)	y=0.992+0.273x	0.818 <i>n.s</i>
NA2	3.239 (2.801-3.616)	7.082 (6.493-7.895)	y=1.080+0.333x	2.307 <i>n.s</i>

LC₅₀ = lethal concentration that kills 50 % of the exposed organisms

LC₉₀ = lethal concentration that kills 90 % of the exposed organisms

χ^2 = chi-square value

d.f. = degrees of freedom

n.s. = not significant ($\alpha=0.05$)