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Neem by-products in the fight against mosquito-borne diseases: Biotoxicity of neem cake fractions towards the rural malaria vector *Anopheles culicifacies* (Diptera: Culicidae)

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ABSTRACT

Objective: To evaluate the ovicidal, larvicidal and adulticidal potential of neem cake fractions of different polarity against the rural malaria vector *Anopheles culicifacies* (*An. culicifacies*).

Methods: Neem cake fractions' total methanol extract (NTMeOH), total ethyl acetate extract (NTAcOEt), ethyl acetate fraction after repartition with NTMeOH (NRAcOEt), butanol fraction after repartition with NTMeOH (NRBuOH), and aqueous fraction after repartition of NTMeOH (NRH_2O) were tested against *An. culicifacies* eggs, fourth instar larvae and adults.

Results: In larvicidal experiments, NTMeOH, NTAcOEt, NRAcOEt, NRBuOH and NRH_2O achieved LC_{50} values of 1.32, 1.50, 1.81, 1.95 and 2.54 mg/L, respectively. All fractions tested at 150 mg/L were able to reduce egg hatchability of more than 50%, with the exception of NTAcOEt and NRAcOEt. In adulticidal assays, NTMeOH, NTAcOEt, NRAcOEt, NRBuOH and NRH_2O achieved LC_{50} values of 3.01, 2.95, 3.23, 3.63 and 3.00 mg/L, respectively.

Conclusions: Overall, this study suggests that the methanolic fractions of neem cake may be considered as a new and cheap source of highly effective compounds against the rural malaria vector *An. culicifacies*.

1. Introduction

According to the latest estimates, there were about 198 million cases of malaria in 2013 and an estimated 584 000 deaths. Malaria mortality rates have fallen by 47% globally since 2000 and by 54% in the African region. Most deaths occur among children living in Africa, where a child dies every minute from malaria [1]. *Anopheles culicifacies* Giles (*An. culicifacies*) is the most important malaria vector in rural and peri-urban areas of Peninsular India, contributing to nearly 65% of total malaria cases per year [2]. *An. culicifacies* is a complex of five sibling species, provisionally designated as A, B, C, D, and E. Among these five, only three species (A, B, and C) have been laboratory-colonized. Members of *An. culicifacies* complex remarkably differ in some behavioral traits, including anthropophilic index, biting rhythm, insecticidal resistance, and vector capacity. In particular, A and C are more competent vectors of *Plasmodium* spp. over B [3]. Mosquito control is a difficult task and is becoming even more so due to a variety of factors, including development of insecticide resistance and concerns

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on environmental pollution [4]. In this scenario, eco-friendly control tools are a priority [5,6].

Among botanical products active against mosquito vectors, neem-based chemicals are of particular interest. Neem, *Azadirachta indica* A. Juss. (Meliaceae), is a pantropical fast-growing tree species. The medical properties of neem have been anciently reported in Indian writings. Sanskrit documents referred to the medical uses of neem fruits, seeds, oil, leaves, roots and bark. Later on, this has been confirmed in the Indian Ayurvedic and Unani systems of medicines. Through the centuries, the medical importance of neem increased, and it is currently appreciated for its importance in the everyday life of Asiatic populations [7,8]. Nowadays, rural Indian populations call the neem tree their "village pharmacy" because it "cures" a wide range of diseases and disorders, ranging from teeth caves and bedbugs to ulcers and malaria. The oil extracted from the neem kernels, commercially known as neem oil or margosa oil, has great commercial utilization as insecticide. The economical importance of neem oil is boosted by the fact that it is the only plant-borne biocide accepted by the U.E. normative (Directive 2012/15/EU). Neem seeds contain more than 200 bioactive chemicals, even if attention has been mainly focused on limonoids (chemically known as nortriterpenes, *e.g.* azadirachtin, nimbin, nimbidin and nimbolide) [8]. Formulations deriving from neem seeds showed antifeedancy, fecundity suppression, ovicidal and larvicidal activity, growth regulation and repellence against a great number of arthropod pests, also at low dosages [9–14].

Neem cake is a waste of the manufacture of neem oil, obtained by cold pressing neem kernels from handpicked and cleaned neem fruits and seeds. India has an annual potential of 80 000 metric tons of neem oil and 330 000 metric tons of neem cake from 0.42 million metric tons of neem seed and 14 million neem trees [8]. For a long time, neem cake has been considered a byproduct of low chemical interest, used in agriculture as fertilizer or as animal feed. Later on, it has been highlighted that the low-cost and abundance of neem cake make it a potential raw material for developing eco-friendly mosquitocidal products [15,16], including ovideterrents against the Asian tiger mosquito, *Aedes albopictus* (*Ae. albopictus*).

In this research, we evaluated the ovicidal, larvicidal, pupicidal and adulticidal properties of neem cake fractions of increasing polarity against the rural malaria vector *An. culicifacies*. High performance liquid chromatography (HPLC) analyses were also conducted to shed light on the main constituents responsible for neem cake's ovicidal, larvicidal and adulticidal activity.

2. Materials and methods

2.1. Fractionation process of neem cake

Neem cake was provided by Neem Italia [Manerba (BS), Italia]. Neem cake (3 kg) was extracted with methanol (3 L) at room temperature, twice for 4 days, obtaining, after evaporation of the solvent, 49 g of the total methanol extract (NTMeOH). The same procedure was repeated using ethyl acetate as solvent, obtaining the total ethyl acetate extract (NTAcOEt). NTMeOH was separated using several solvents, in order to obtain fractions of increasing polarity. NTMeOH was defatted by *n*-hexane treatment, obtaining by filtrating the *n*-hexane fraction. The defatted residue was partitioned between equal quantities of water and ethyl acetate (1:l), obtaining two phases, a second organic fraction [ethyl acetate fraction after repartition with NTMeOH (NRAcOEt), 22 g], and an aqueous fraction. The aqueous fraction was partitioned with an equal quantity of *n*-butanol, obtaining a third organic fraction [butanol fraction after repartition with NTMeOH (NRBuOH), 5 g] and the final aqueous fraction [aqueous fraction after repartition of NTMeOH (NRH_2O)]. All fractions were tested for their biological activity and examined by HPLC. In order to ascertain the neem cake identity in the raw material, 5 g of NTAcOEt were separated by column chromatography on Si gel in toluene/ethyl acetate (9:1), obtaining four fractions of approximately 1 g each. Part of the less polar fraction was further separated by column chromatography in the above conditions, obtaining eight fractions (I–VIII). Fraction V (51 mg) contained a pure product that was identified as salannin by nuclear magnetic resonance. Further information was obtained by HPLC analysis.

2.2. HPLC analyses

HPLC measurements were carried out on a Perkin Elmer LC apparatus (Perkin Elmer Corporation, Sheldon, CT, USA). Binary Series 200 Pump, Series 200 UV–vis fixed wavelength detector, and NCI 900 PE Nelson Chromatography Interface linked to a PC were used. Data acquisition was done with Turbochrom version 6.2.0 software. Injection volume loop was 20 µL. Stationary phase was as follows: Restek C18 II Pinnacle, 250 mm × 46 mm, 5 µm particles (Restek, USA); flow rate 1.00 mL/min; UV–vis detector 214 nm; elution program 8 min isocratic, 45% CH_3CN/55% water; 22 min linear gradient to 100% CH_3CN; 10 min isocratic 100% CH_3CN. Retention times were as follows: azadirachtin A 6.2 min, nimbin 21.6 min and salannin 22.4 min. All assignments were confirmed by conjunction with the standard solutions. For quantitative analyses, calibration curves were drawn for all of the species of interest, using standard solutions in the 1–10 mg/L range. Azadirachtin A (97%), salannin (96%) and nimbin (96%) standards were from Trifolio-M GmbH (Lahnau, Germany). Standard solutions of 1 000 mg/L of each compound were obtained by solution of the adequate quantity in 1 mL methanol. Further comparisons were obtained using diractin (Serbios, Rovigo, Italia), with a total azadirachtin content of 32 g/L. Solvents were H_2O "HPLC grade" and methanol "RG grade" were from Baker (Mallinckrodt Baker B.V., Deventer, Olanda), CH_3CN "HPLC grade" were from Biosolve (Biosolve B.V., Valkenswaard, Olanda).

2.3. An. culicifacies rearing

Eggs and larvae of *An. culicifacies* were collected from Department of Rice, All India Co-ordinated Research Project, Rice Research Centre (Tamil Nadu Agricultural University, Coimbatore, India). Following the method reported by Murugan *et al.* [17], the eggs were transferred to laboratory conditions [(27 ± 2) °C, 75%–85% relative humidity, 14:10 (light: dark) photoperiod] and placed in 18, 9, 13, 9, 4 cm plastic containers containing 500 mL of tap water, waiting for larval hatching. Larvae were reared in the plastic containers described above, and fed daily with a mixture of crushed dog biscuits (Pedigree, USA) and hydrolyzed 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 for experiments were collected daily from

culture containers and transferred to glass beakers containing 500 mL of water [2,18].

2.4. Experimental concentrations

One gram of the each neem cake fraction was separately dissolved in 100 mL of acetone (stock solution) and considered as 1% stock solution. From this stock solution, different mosquitocidal concentrations were prepared ranging from 0.5 to 2.5 mg/L, 1.5–7.5 mg/L, and 50–550 mg/L, respectively for larvicidal, ovicidal and adulticidal assays.

2.5. Larvicidal toxicity

A laboratory-reared colony of *An. culicifacies* larvae was used for the larvicidal activity. Twenty-five individuals of fourth instar larvae were kept in a 500 mL glass beaker containing 249 mL of dechlorinated water and 1 mL of the desired concentration (0.5, 1, 1.5, 2 and 2.5 mg/L) of neem cake fractions (NTMeOH, NTAcOEt, NRAcOEt, NRBuOH and NRH_2O). For each tested concentration, five replicates were carried out. The larvae exposed to dechlorinated water mixed with the same amount of the tested solvent served as control. Control mortalities were corrected by using Abbott's formula (Abbott 1925):

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

Then, mortality rates were calculated as follows:

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

2.6. Ovicidal activity

Following Su and Mulla [19], *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). For each mosquito species, the eggs were placed in a cage with six glass cups (diameter: 6 cm). Five of them were filled with water plus a neem cake fraction treatment (50, 150, 250, 350 and 450 mg/L). The control cup was filled with distilled water. A total of 100 eggs were placed in each cup. Five replicates were done for each tested 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 [20]. The hatch rates were assessed 48 h post-treatment using the following formula [21]:

$$\text{Egg mortality (\%)} = (\text{Number of hatched larvae/Total number of eggs}) \times 100$$

2.7. Adulticidal activity

Adulticidal bioassays were performed following the World Health Organization method [22]. Neem cake fractions were tested at 1.5, 3.0, 4.5, 6.0 and 7.5 mg/L, and 2 mL were applied on Whatman No. 1 filter paper (size 12 cm × 15 cm) lining a glass holding tube (diameter 30 mm; length 60 mm). Control filter paper was treated with distilled water respectively. 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 and kept for a 24 h recovery period. A pad of cotton soaked with 10% (w/w) glucose solution was placed on the mesh screen at the top of the holding tube.

2.8. Data analysis

Egg mortality data were checked for normality and subjected to Two-way ANOVA (*i.e.* factors: tested fraction and dose). Means were separated using Tukey's honest significant difference test. Results with $P < 0.05$ were considered to be statistically significant. Larvicidal and adulticidal data were subjected to probit analysis. LC_{50} and LC_{90} values were calculated following the method by Finney (1971). SPSS (statistical software package) 16.0 version was used.

3. Results

Considering 50 mg/L of neem cake whole extract, results of HPLC analysis were azadirachtin A 0.7 mg/L, nimbin 0.3 mg/L and salannin 1.5 mg/L. In larvicidal experiments against *An. culicifacies*, NTMeOH, NTAcOEt, NRAcOEt, NRBuOH and NRH_2O achieved LC_{50} values of 1.321, 1.504, 1.818, 1.950 and 2.545 mg/L, respectively (Table 1). Concerning ovicidal potential, all fractions tested at 150 mg/L were able to reduce egg hatchability of more than 50%, with the exception of NTAcOEt and NRAcOEt (Table 2). In adulticidal assays, NTMeOH, NTAcOEt, NRAcOEt, NRBuOH and NRH_2O achieved LC_{50} values of 3.015, 2.954, 3.239, 3.637 and 3.003 mg/L, respectively (Table 3). Within an hour of exposure, *An. culicifacies* adults showed restless movement, with abnormal wagging, and then died.

Table 1
Larvicidal activity of neem cake fractions of increasing polarity against the rural malaria vector *An. culicifacies*.

Treatment	LC_{50} (mg/L) (95% LCL–UCL)	LC_{90} (mg/L) (95% LCL–UCL)	Regression equation	χ^2 ($df = 4$)
NTMeOH	1.321 (1.100–1.516)	3.517 (3.037–4.349)	$y = 0.771 + 0.584x$	$0.575^{n.s.}$
NTAcOEt	1.504 (1.271–1.739)	4.031 (3.388–5.246)	$y = 0.763 + 0.507x$	$0.598^{n.s.}$
NRAcOEt	1.818 (1.559–2.172)	4.773 (3.867–6.711)	$y = 0.789 + 0.434x$	$0.657^{n.s.}$
NRBuOH	1.950 (1.675–2.373)	5.020 (4.023–7.231)	$y = 0.814 + 0.417x$	$0.307^{n.s.}$
NRH_2O	2.545 (2.154–3.372)	5.870 (4.560–9.081)	$y = 0.981 + 0.385x$	$0.223^{n.s.}$

LC_{50}: Lethal concentration that kills 50% of the exposed organisms; LC_{90}: Lethal concentration that kills 90% of the exposed organisms; LCL: Lower confidence limit; UCL: Upper confidence limit; χ^2: *Chi*-square ($\alpha = 0.05$); $^{n.s.}$: Not significant.

Table 2
Ovicidal activity of neem cake fractions of increasing polarity against the rural malaria vector *An. culicifacies*. Mean ± SD.

Treatment	% Egg hatchability						
	Control	50 mg/L	150 mg/L	250 mg/L	350 mg/L	450 mg/L	550 mg/L
NTMeOH	100.0 ± 0.0^{a}	62.3 ± 1.4^{b}	48.4 ± 1.1^{c}	25.6 ± 1.0^{d}	NH	NH	NH
NTAcOEt	100.0 ± 0.0^{a}	66.3 ± 1.6^{b}	52.9 ± 1.3^{c}	28.5 ± 1.6^{d}	NH	NH	NH
NRAcOEt	100.0 ± 0.0^{a}	76.5 ± 1.0^{b}	59.5 ± 1.7^{c}	40.7 ± 1.4^{d}	NH	NH	NH
NRBuOH	100.0 ± 0.0^{a}	45.4 ± 1.9^{b}	31.2 ± 0.7^{c}	12.4 ± 1.2^{d}	NH	NH	NH
NRH_2O	100.0 ± 0.0^{a}	50.9 ± 1.3^{b}	23.9 ± 1.7^{c}	10.8 ± 1.8^{d}	NH	NH	NH

Within each row, different superscript letters indicate significant differences (Tukey's honest significant difference, $P < 0.05$). NH: No hatchability.

Table 3
Adulticidal activity of neem cake fractions of increasing polarity against the rural malaria vector *An. culicifacies*.

Treatment	LC_{50} (mg/L) (95% LCL–UCL)	LC_{90} (mg/L) (95% LCL–UCL)	Regression equation	χ^2 ($df = 4$)
NTMeOH	3.015 (1.452–3.967)	6.481 (5.290–9.501)	$y = 1.115 + 0.370x$	8.808$^{n.s.}$
NTAcOEt	2.954 (0.743–4.088)	6.666 (5.276–11.114)	$y = 1.020 + 0.345x$	11.126*
NRAcOEt	3.239 (2.801–3.616)	8.334 (7.521–9.532)	$y = 1.080 + 0.333x$	0.818$^{n.s.}$
NRBuOH	3.637 (3.141–4.070)	7.279 (6.716–8.040)	$y = 0.992 + 0.273x$	0.598$^{n.s.}$
NRH_2O	3.003 (0.801–4.135)	7.601 (6.997–8.429)	$y = 0.878 + 0.292x$	1.087$^{n.s.}$

LC_{50}: Lethal concentration that kills 50% of the exposed organisms; LC_{90}: Lethal concentration that kills 90% of the exposed organisms; LCL: Lower confidence limit; UCL: Upper confidence limit; χ^2: *Chi*-square ($\alpha = 0.05$); $^{n.s.}$: Not significant; *: Indicates $P < 0.05$.

4. Discussion

HPLC analysis highlighted that azadirachtin A, nimbin and salannin were the major constituents of neem cake whole extract. In agreement with our findings, neem cake fractions obtained from different producers have been recently extracted with solvents of different polarities and analyzed using high performance thin layer chromatography (HPTLC). HPTLC highlighted the presence of several limonoids in neem cake extracts, with prevalence of salannin (in *n*-hexane, methanol and ethyl acetate extracts) and nimbin (hexane extract) [16,23].

To the best of our knowledge, this is the first report about neem cake biotoxicity against young instars of the rural malaria vector *An. culicifacies*. However, several researches showed that neem cake fractions could be effective as oviposition repellents against *Ae. albopictus* in field conditions and act as oviposition inhibitors against *An. culicifacies* adults [9,23]. In our ovicidal experiments, all fractions tested at 150 mg/L were able to reduce egg hatchability of more than 50%, with the exception of NTAcOEt and NRAcOEt. The ovicidal action may be linked with the presence of azadirachtins, which previously showed complete ovicidal activity against eggs of *Culex tarsalis* and *Culex quinquefasciatus* (*Cx. quinquefasciatus*) exposed to 10 mg/L [24]. Furthermore, Rao *et al*. reported that neem cake powder applied in rice fields at a dose of 500 kg/ha, either alone or coated over urea, has been able to exert a strong reduction in the abundance of *Cx. quinquefasciatus* late-instar larvae and pupae [25]. Shanmugasundaram *et al*. tested neem cake against fourth-instar larvae of *Cx. quinquefasciatus*, *Ae. aegypti* and *Anopheles stephensi* and reported good toxic properties (LC_{50} = 0.56% (w/v), 0.29% and 0.45%, respectively) [26]. Later on, Nicoletti *et al*. studied the bioactivity of neem cake fractions of increasing polarity (dosage: 50 mg/L) against eggs of *Ae. albopictus*, showing no differences in egg hatching over control [15]. When newly emerged larvae were allowed to develop in the neem cake solutions, higher mortality rates have been reported after 8 days for hexane and ethyl acetate fractions, over butanol fraction, aqueous fraction and control. In addition, the neem cake methanol extract was able to block surviving *Ae. albopictus* individuals at larval stages [15]. Nicoletti *et al*. extended the neem cake bioactivity survey against the Asian tiger mosquito, testing six commercial samples, and reported significant differences in toxicity exerted by different neem cake samples against *Ae. albopictus* larvae [16]. Three samples did not show significant mosquitocidal activity on newly hatched larvae, and two of them were not toxic towards late-instar larvae [16]. It has been argued that the observed differences in mosquito larvicidal activity over different neem cake products can be partially due to the given amounts of several minor constituents that are able to synergize the insecticidal effect of major chemicals [8].

The chemical composition of neem cake is quite different from the oil. Previous research, based on HPTLC fingerprint in order to evidence the total metabolic production, showed a complex composition, which can be variable according to raw material origin, methods of extraction and purification of the oil, the conservation procedures. Identity of neem cake is important in progress for its utilization, since several differences are present in marketed neem cakes, as well as the oil [14–16,23]. Overall, the results of this study, showing that methanol extract of neem cake of *Azadirachta indica* possesses larvicidal, adulticidal and ovicidal effects against the malarial vector *An. culicifacies*, confirm the potentiality of neem cake in the development and potential of new alternative sources to build cheap and safer insecticides against vectors of mosquito-borne diseases. Further research is in progress and will focus on the chemical determination of active constituents, besides azadirachtins, to enhance stability of neem commercial products.

Conflict of interest statement

We declare that we have no conflict of interest.

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