Larvicidal and ovideterrent properties of neem oil and fractions against the filariasis vector *Aedes albopictus* (Diptera: Culicidae): a bioactivity survey across production sites

Giovanni Benelli, Stefano Bedini, Francesca Cosci, Chiara Toniolo, Barbara Conti, Marcello Nicoletti

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Corresponding Author:	Giovanni Benelli, Ph.D. University of Pisa Pisa, ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Pisa
Corresponding Author's Secondary Institution:	
First Author:	Giovanni Benelli, Ph.D.
First Author Secondary Information:	
Order of Authors:	Giovanni Benelli, Ph.D.
	Stefano Bedini, Ph.D.
	Francesca Cosci, M.D.
	Chiara Toniolo, Ph.D.
	Barbara Conti, Ph.D.
	Marcello Nicoletti, Professor

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Larvicidal and ovideterrent properties of neem oil and fractions against the filariasis vector *Aedes albopictus* (Diptera: Culicidae): a bioactivity survey across production sites

Giovanni Benelli[1 §], Stefano Bedini[1], Francesca Cosci[1], Chiara Toniolo[2], Barbara Conti[1 *], Marcello Nicoletti[2]

[1] Department of Agriculture, Food and Environment, University of Pisa, via del Borghetto 80, 56124 Pisa, Italy

[2] Department of Environmental Biology, University Sapienza of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

[§] Giovanni Benelli. Tel.: +390502216141. Fax: +390502216087. E-mail addresses: g.benelli@sssup.it; benelli.giovanni@gmail.com

* Corresponding author: Barbara Conti. Tel.: +390502216125. Fax: +390502216087. E-mail: barbara.conti@unipi.it

Abstract

Neem seed oil (NSO) of *Azadirachta indica* (Meliaceae) contains more than one hundred determined biologically active compounds, and many formulations deriving from them showed toxicity, antifeedancy and repellence against a number of arthropod pests. However, it is widely known that botanical products can differ in their chemical composition and bioactivity, as function of the production site and production process. We used HPTLC (High Performance Thin Layer Chromatography) to investigate differences in chemical constituents of NSOs from three production sites. HPTLC analyses showed several differences in chemical abundance and diversity among NSOs, with special reference to limonoids. Furthermore, the three NSOs and their fractions of increasing polarities [i.e. ethyl acetate fraction (EA) and butanol fraction (BU)] were evaluated for larvicidal toxicity and field oviposition deterrence against the Asian tiger mosquito, *Aedes albopictus*, currently the most invasive mosquito worldwide. Results from bioactivity experiments showed good toxicity of NSOs and EA fractions against *A. albopictus* fourth instar larvae (with LC_{50} values ranging from 142.28 to 209.73 ppm), while little toxicity was exerted by BU fractions. A significant effect of the production site and dosage was also found, and is probably linked to differences in abundance of constituents among samples, as highlighted by HPTLC analyses. NSOs and EAs were also able to deter *A. albopictus* oviposition in the field (effective repellence values ranging from 98.55 % to 70.10%), while no effectiveness of BU fractions was found. Concerning ovideterrent activity, no difference due to the production site was found. This is the first report concerning larvicidal toxicity of NSO against *A. albopictus* and ovideterrence against Culicidae in the field. The chance to use chemicals from the NSO EA fraction seems promising, since they are effective at lower doses, if compared to synthetic products currently marketed, and could be an advantageous alternative to build newer and safer mosquito control tools.

Key words: arbovirus vector; Asian tiger mosquito; botanical by-products; *Azadirachta indica*; HPTLC; Meliaceae; plant-born mosquitocidals

Introduction

Since the Middle Ages, plant-borne compounds, such as essential oils and extracts, have been employed for bactericidal, virucidal, fungicidal, parasiticide and insecticidal applications (Amer and Mehlhorn 2006a). After a period of synthetic products dominancy, in the last two decades, renewed efforts have been done to investigate the bioactivity of new plant-borne compounds against an impressive range of arthropod pests, including tephritid flies (Benelli et al. 2012a, 2013a; Canale et al. 2013), foodstuff beetles (Benelli et al. 2012b) and parasites of medical and veterinary importance, with a special focus on mosquitoes (Elango et al. 2011; Conti et al. 2012a; Conti et al. 2013; Giatropoulos et al. 2013; Panneerselvam and Murugan 2013; Conti et al. 2014). Many plant essential oils and extracts have been recognised as excellent Culicidae ovicidal (Govindarajan et al. 2011), larvicidal (Amer and Mehlhorn 2006a; Hafeez et al. 2011; Benelli et al. 2013b), adulticidal (Govindarajan et al. 2012; Panneerselvam et al. 2012), growth and/or reproduction inhibitors (Rajkumar and Jebanesan 2005; Pushpanathan et al. 2006), adult repellents (Amer and Mehlhorn 2006b; Koliopoulos et al. 2010; Gleiser et al. 2011; Conti et al. 2012b) and oviposition deterrents (Xue et al. 2001; Elango et al. 2009; Rajkumar and Jebanesan 2009). Their use has to face problems of production, formulation, stability and costs. However, natural products still represent one of the most promising possibilities to explore new eco-friendly solutions against mosquitoes.

Among Culicidae, the Asian tiger mosquito, *Aedes albopictus* (Skuse), is actually acknowledged as the most invasive mosquito species in the world (Benedict et al. 2007; Caminade et al. 2012), due to its ecological and physiological plasticity (Yamany et al. 2012). Environmental impact of Asian tiger mosquito is expanding in several countries sustained by climate changes (Reichter 2001; Nicoletti et al. 2014). The medical importance of *A. albopictus* is mainly due to the aggressive daytime human-biting behaviour and to its ability to transmit many viruses, including dengue, yellow fever, West Nile and chikungunya. It also acts as a vector of filariasis, with special reference to *Dirofilaria immitis* Leidy, *Dirofilaria repens* Railliet & Henry and *Setaria labiatopapillosa* Perroncito (Benedict et al. 2007; Paupy et al. 2009). Unfortunately, there are no vaccines or effective drugs against the main pathogens and

parasites transmitted by *A. aldopictus*, and vector control remains a pivotal prevention tool. Although *A. aldopictus* larvae can be killed by organophosphates and insect growth regulators, there is an raising number of resistant mosquito strains (Hemingway and Ranson 2000). Biological control tools, based on the release of larvivorous organisms, are frequently not suitable in the majority of urban environments exploited by *A. albopictus* larvae and still require further research (Bowatte et al. 2013). Therefore, there is an urgent need to find safer and eco-friendly alternatives to enhance the Asian tiger mosquito control strategies.

The neem tree, *Azadirachta indica* A. Juss (Meliaceae), is a fast growing evergreen tree native of Indian subcontinent and valued as an important source of eco-friendly phytochemicals for human health and pest management (National Research Council 1992). The main product of neem is the oil extracted from its seeds. Neem seed oil (NSO) contains at least one hundred biologically active compounds. Among them, major constituents are nor-triterpenes, named limonoids, i.e. azadirachtin, nimbin, nimbidin and nimbolides. NSO is obtained by different extraction methods. Most of the NSO is produced in India by familiar small producers, but many other countries are now producing NSOs. Therefore, considering also the possible different geographical origin of the raw material, combined pre- and post-harvesting factors can result in great differences in constituents present in marketed NSOs, as recently reported by Gallo et al. (2012).

Many formulations deriving from neem seeds show antifeedancy, fecundity suppression, ovicidal and larvicidal activity, insect growth regulation and/or repellence against insect pests, even at low dosages (Dua et al. 2009; Egho 2012). For instance, the concentrate extract of neem seeds [e.g. MiteStop, developed by the University spin-off company Alpha-Biocare (Düsseldorf, Germany)] is effective against a number of pests of medical and veterinary importance (Semmler et al. 2010), including ticks, house dust mites, cockroaches, raptor bugs, cat fleas, bed bugs (Schmahl et al. 2010), biting and bloodsucking lice (Al-Quraishy et al. 2011; Al-Quraishy et al. 2012; Abdel-Ghaffar et al. 2012; Mehlhorn et al. 2012), *Sarcoptes scabiei* De Geer mites infesting dogs (Abdel-Ghaffar et al. 2008), poultry mites (Abdel-Ghaffar et al. 2009; Locher et al. 2010) and beetle larvae parasitizing the plumage of poultry (Walldorf et al. 2012). Other advantages arising from the use of neem-based products are no induction of resistance,

due to their multiple mode of action against pests and low toxicity rates against vertebrates (Nicoletti et al. 2010, 2012). Overall, the insecticidal properties, environmental safety and public acceptability of neem and its products for the control of insect pests has led to its adoption into some control programs against Diptera pests (Sharma and Dhiman 1993; Su and Mulla 1998a, b), despite some limitations including the relatively high cost of refined products and the low persistence on treated surfaces exposed to sunlight (Isman 2006). Noticeably, emulsified formulations of *A. indica* oil showed an excellent larvicidal potential against different mosquito genera, including *Aedes, Anopheles* and *Culex*, also under field conditions (Dua et al. 2009). However, it is widely recognised that botanical products can differ in their chemical composition and bioactivity against a targeted pest as function of the plant's geographical origin and cultivation technique (Tchoumbougang et al. 2005; Noudjou et al. 2007; Conti et al. 2013). Variability in bioactivity has been reported also for neem products (Koul et al. 1990; Gallo et al. 2012; Hashmat et al. 2012).

In this study, we used HPTLC (High Performance Thin Layer Chromatography) to investigate differences in metabolic constituents of three NSOs from different production sites (two from India and one from Thailandia). NSOs and their fractions of increasing polarities [i.e. ethyl acetate fraction (EA) containing the less polar substances, and the butanol fraction (BU), mainly composed by constituents of medium polarity] were then evaluated for their larvicidal toxicity and ovideterrent properties.

Materials and methods

Neem samples

Samples NSO1 and NSO2 were kindly provided by industrial producers that use them as raw material for preparation of their products. NSO1 was neem seed oil from plants cultivated in India by an industrial producer; NSO2 = was neem seed oil from plants cultivated in Thailandia by an industrial producer. NSO3 was directly collected by one of the Authours (M.N.) from a small local producer in Bangalore (South-East of India). Standards used in the HPTLC analysis

were isolated from neem oil and neem cake (i.e. salannin, azadirachtin A, unsaturated and saturated lipids) in previous research (Nicoletti 2011).

HPTLC system, materials and sample application

HPTLC is the last evolution of planar chromatography (Reich and Schibli 2007; Gallo et al. 2012). Allowing to the capacity in evidencing natural products, including also those in very low concentrations, HPTLC is used to perform metabolome studies, like determination of most of the constituents of an extract (Ram et al. 2011; Gallo et al. 2011). Main product of HPTLC analysis is the chromatographic fingerprint, consisting in the individual track typical of the extract or the product. Chromatographic fingerprint analytic approach received important official recognition (WHO 2000; AOAC 2005; Chinese Pharmacopoeia 2009). Plates can be visualized and derivatised in several ways, obtaining multiple information, as well as converted in a series of peaks by densitometric treatment. In such way, the comparison between samples is reliable and facilitated by the visual inspection and samples can be analysed side-by-side and exactly in the same conditions (Gallo et al. 2012). Here, HPTLC was selected to investigate the differences in composition of the tested NSOs and to obtain information about chemical nature of active constituents. Determination of single chemicals (e.g. salannin, azadirachtin A, nimbin) was also achieved by direct comparison with selected standards, obtained in previous research (Nicoletti et al. 2012). Limonoids standards concentration was 2 mM.

The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of a Linomat 5 sample applicator using 100 µL syringes and connected to a nitrogen tank; a chamber ADC 2 containing twin trough chamber 20 x 10 cm; an immersion device III; a TLC Plate Heater III; a TLC visualizer; a TLC scanner 3 linked to win CATS software.

Solvents for extraction and HPTLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy). Glass plates 20 cm x 10 cm with glass-backed layers silica gel 60 (2 µm thickness) were from Merck (Darmstadt, Germany). Before use, plates were prewashed with methanol and dried for 3 min at 100 °C. Standards used in HPTLC were isolated in previous researches (Benelli et al. 2014b). Filtered solutions were applied with

nitrogen flow. The operating conditions were: syringe delivery speed, 10 s μL^{-1} (100 nL s^{-1}); injection volume, 2 µL; band width, 6 mm; distance from bottom, 15 mm.

Development of HPTLC plates

The HPTLC plates were developed using the solvent system toluene: ethyl acetate 7:3 (v/v) as mobile phase, in the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase for 20 min at room temperature. The developing solvents (i.e. type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 80 mm from the point of application. The developed layers were allowed to dry in air for 5 min and then derivatized with a selected solution, including anhysaldehyde (1.5 mL *p*-anisaldehyde, 2.5 mL H_2SO_4, 1 mL AcOH in 37 mL EtOH) and/or Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate), dried in the open air and then dipped into Macrogol reagent (1 g polyethylene glycol 400 in 20 mL of dichloromethane). Finally, the plates are warmed for 5 min at 120 °C before inspection. All treated plates were then inspected under a UV light at 254 and 366 nm or under reflectance and transmission white light (WRT), respectively, at a Camag TLC visualizer, before and after derivatization. Phenolic nature of compounds at the starting line was confirmed by positive reaction at ferric chloride and ferricyanide tests (Marini-Bettolo et al. 1981; Graham 1992).

Validation, stability and repeatability

Band stability and overlapping of bands is a typical analytical challenge for complex mixtures like multi-ingredient products. HPTLC allowed a good separation and visualization of the constituents. Sample solutions were found to be stable at 4 °C for at least 1 month and for at least 3 days on the HPTLC plates.

Repeatability was determined by running a minimum of three analyses. Rf values of main selected compounds varied ± 0.02%. The effects of small changes in the mobile phase composition, mobile phase volume and duration of saturation were minute and reduced by the

direct comparison. Results were critically dependent on prewashing of HPTLC plates with methanol (Benelli et al. 2014b).

Fractionation process of NSO samples

Fractions of different NSO samples were obtained by repartition of the dry methanol extract of the oil between H_2O and ethyl acetate (1:1, v/v), obtaining the EA together with the aqueous phase. The aqueous phase was retained and partitioned adding an equal volume of *n*-BuOH. Then, BU was obtained (Benelli et al. 2014b).

Larvicidal activity

Three groups of twenty fourth-instar larvae were isolated in 250 mL beakers and exposed for 24 hours days to 100, 150 or 200 ppm of the following chemicals: NSOs 1-3, and their respective ethyl acetate and butanol fractions. Each tested product was dissolved in tap water containing 0.1% of Tween 80. Tap water with 0.1% of Tween 80 was used as control. Mortality was checked after 24 h. Larval mortality was reported as an average of three replicates (WHO 2009; Benelli et al. 2014a). Since no mortality was registered in the control treatment, the mortality percentage rates were not corrected (Benelli et al. 2013b).

Oviposition deterrence in the field

Oviposition deterrence of the following chemicals: NSOs 1-3, and their respective ethyl acetate and butanol fractions, was evaluated in the open field. Experiments were carried out in the garden (about 3000 m^2) of the entomology laboratory at the Department of Agriculture, Food and Environment, University of Pisa (Italy). All experiments were conducted from June to August 2014.

Methods described by Xue et al. (2001) and Benelli et al. (2014b) for the oviposition deterrence field tests were followed. Chemicals were tested at the concentration of 200 ppm,

dissolved in tap water containing 0.1% of Tween 80. For each treatment, three black plastic containers (10 x 10 x 12 cm) holding 500 mL of water plus the tested compound were placed outdoors. The three containers were arranged in lines and separated by 50 mm each other. A brown masonite ovistrip (200 x 25 mm) was placed in each of the three containers.

For each test, a control treatment was run placing a group of control containers in proximity (about 80 cm) of the group of treated oviposition containers. All control treatments contained tap water with 0.1% of Tween 80. Both for treatments and controls, the positions of the container were alternated between the different replicates, to avoid oviposition bias due to positional effects (Benelli et al. 2014b).

Masonite ovistrips were checked daily. After seven days, they were removed, air dried in the laboratory and the number of *A. albopictus* eggs laid in treated and control ovistrips was counted using a stereomicroscope (Leica, Germany). The percent effective repellence for each concentration was calculated using the following formula (Rajkumar and Jebanesan 2009; Benelli et al. 2014b):

$$ER\% = [(NC - NT) / NC] * 100$$

Oviposition activity index (OAI) was calculated using the formula (Cheah et al. 2013; Benelli et al. 2014b):

$$OAI = (NT - NC) / (NT + NC)$$

NT = total number of eggs in the test solution and NC = total number of eggs in the control solution.

Oviposition active index of +0.3 and above are considered as attractants while those with −0.3 and below are considered as repellents (Kramer and Mulla 1979). Positive values indicate that more eggs were deposited in the test containers than in the control containers and that the test solutions were attractive. Conversely, negative values indicate that more eggs were deposited in the control containers than in the test containers and that the test solutions were a

deterrent (Cheah et al. 2013; Benelli et al. 2014b).

Data analysis

Larval mortality data were transformed into arcsine√proportion values before statistical analysis. Data were processed with JMP, using a General Linear Model (GLM) with three factors, the neem geographical origin, the tested chemical (oil, ethyl acetate fraction and butanol fraction) and the dosage (100, 150 or 200 ppm): $y_j = \mu + O_j + C_j + D_j + O_j*C_j + O_j*D_j + C_j*D_j + O_j*C_j*D_j + e_j$, in which y_j is the observation, μ is the overall mean, O_j the origin (j = 1-3), C_j the chemical (j = 1-3), D_j the dosage (j = 1-3), O_j*C_j the interaction origin*chemical, O_j*D_j the interaction origin*dosage, C_j*D_j the interaction chemical*dosage, $O_j*C_j*D_j$ the interaction origin*chemical*dosage, and e_j the residual error. Averages were separated by Tukey-Kramer HSD test. $P < 0.05$ was used for the significance of differences between means.

Median lethal dose (LD_{50}) against Asian tiger mosquito larvae was calculated by Log-probit regressions by the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Significant differences between LD_{50} values were determined by estimation of confidence intervals of the relative median potency. Differences among LD_{50} values were judged as statistically significant when values in the 95% confidence interval of relative median potency analyses were ≠ 1.0.

Effective oviposition deterrence percentage data were transformed into arcsine√proportion values, before statistical analysis. Effective oviposition deterrence percentage data were processed with JMP, using the above-described GLM with two factors (the neem origin and the tested chemical) and their interaction. Averages were separated by Tukey-Kramer HSD test. $P < 0.05$ was used for the significance of differences between means.

Results

HPTLC and densitometric analyses

Compositions of NSOs from different production sites were compared. Metabolites spots,

diffused in the tracks according to the polarity of constituents, showed similarities of fingerprints NSO 1 and 2 (**Figure 1**). Identification of raw material was assured by the presence of salannin (Rf = 0.42), a typical maker of NSO. In comparison with the spot of azadirachtin A (Rf = 0.23), salannin appeared the main limonoid spot. Spots concerning lipids were present at Rf values, at ca. 0.80, due to unsaturated fatty acids and alcohols, and at Rf ca. 0.50, due to saturated and unsaturated triglycerides (based on NMR data, dataset available under request). The most interesting feature of the plates was the presence of compounds with high fluorescent reaction at between Rf 0.55-0.66, that were visible in NSO samples at 366 nm, both before and after derivatization (**Figures 1 and 2**).

Larvicidal activity

NSOs exhibited dose-dependent toxic activity against *A. albopictus* larvae (**Figure 3**). We found a significant effect of the production site ($F = 21.342$; $d.f. = 2$; $P < 0.001$), tested chemical ($F = 60.854$; $d.f. = 2$; $P < 0.001$), dose ($F = 157.478$; $d.f. = 2$; $P < 0.001$), and the interactions production site*chemical ($F = 13.951$; $d.f. = 4$; $P < 0.001$), chemical *dose ($F = 6.501$; $d.f. = 4$; $P < 0.001$), production site*dose ($F = 14.271$; $d.f. = 4$; $P < 0.001$), while the interaction production site*chemical*dose was not significant ($F = 1.578$; $d.f. = 8$; $P = 0.153$).

LC_{50} values were similar among the tested NSOs, as well as among EAs (**Table 1**). However, this was not true for BUs, where LC_{50} showed significant differences according to different producers (**Table 2**, see also **Figure 3**).

Oviposition deterrence

NSOs tested at 200 ppm were able to deter oviposition in *A. albopictus* (**Table 3**). Results showed a significant effect of tested chemical ($F = 41.750$; $d.f. = 2$; $P < 0.001$), while the effects of production site ($F = 2.345$; $d.f. = 2$; $P < 0.125$) and the interaction production site*chemical were not significant ($F = 1.043$; $d.f. = 4$; $P < 0.413$).

ER and OAI showed comparable efficacy of NSO1 (ER = 91.64 ± 5.22; OAI = -0.871),

NSO2 (ER = 70.39 ± 21.20; OAI = -0.514) and NSO3 (ER = 70.10 ± 3.82; OAI = -0.656). Also EA fractions showed comparable ER (94.87 ± 3.24 vs. 91.08 ± 5.67 vs. 98.55 ± 1.45, respectively) and OAI (-0.871 vs. -0.894 vs. -0.984) values (**Table 3**). By contrast, BU fractions were less effective in inducing ovideterrence in *A. albopictus* females, as observed in BU fraction of NSO1 (ER = 41.54 ± 20.60; OAI = -0.245), BU fraction of NSO2 (10.35 ± 3.97; OAI = -0.047) and BU fraction of NSO3 (ER = 4.39 ± 2.16; OAI = -0.018) (**Table 3**).

Discussion

HPTLC analyses highlighted that a number of spots in fingerprint tracks were attributed to nor-triterpenoids and fatty constituents, while several others were related to high conjugated unsaturated aromatic structures, actually under study. Their structures are really different from those of limonoids, which so far have been considered as responsible of bioactivity (Nicoletti et al. 2012). Results from bioactivity experiments showed that NSOs and its fractions were able to exert significant toxicity against *A. albopictus* fourth instar larvae, and the effect was dose-dependent. Previous researches highlighted the toxicity of neem oil against larvae of several Culicidae species. For instance, application of 5% neem oil-water emulsion at 50 mL/m^2 in pools lead to 100% and 51.6% reduction of III-IV instar larvae of *Anopheles stephensi* Liston and *Culex quinquefasciatus* Say after 24 h. Moreover, application of 10% emulsion in desert coolers against *Aedes aegypti* (L.) at dosages ranging from 40 to 80 mL/cooler resulted in complete inhibition of pupal production (Batra et al. 1998). NSO from seeds cultivated in costal Kenya showed very good larvicidal properties against *Anopheles gambiae* Giles (LC_{50} = 11 ppm) (Okumu et al. 2007), while LC_{50} of an industrial neem oil formulation (i.e. neem oil coformulated with polyoxyethylene ether, sorbitan dioleate and epichlorohydrin) against *A. stephensi, C. quinquefasciatus* and *A. aegypti* were 1.6, 1.8 and 1.7 ppm respectively (Dua et al. 2009). Our data extend this survey to *A. albopictus*, showing good larvicidal activity of NSOs and EAs against *A. albopictus* fourth instar larvae (with LC_{50} values ranging from 142.28 to 209.73 ppm). The production site has noticeable impact on NSO toxicity against insect pests, as already found for other plant species (Perry et al. 1999; Santos-Gomes and Fernandes-Ferreira 2001;

Tchoumbougang et al. 2005; Noudjou et al. 2007; see also Conti et al. 2013). Biotoxicity of NSOs against mosquitoes seems mainly due to constituents of EAs, while BUs showed little or no effectiveness against *A. albopictus* larvae. Lastly, toxicity against larvae of *A. albopictus* has been recently validated also testing neem-cake, a by-product of NSO extraction, still rich in limonoids (Rao et al. 1992; Nicoletti et al. 2010; Benelli et al. 2014a).

All tested NSOs, as well as their EAs, were able to deter oviposition of *A. albopictus* females in the field, while the scarcity of chemicals in NSO BU fractions lead to poor efficacy. Compounds tested in our experiments were effective at low dosages (200 ppm). This is the first report about ovideterrent activity of neem oil against mosquitoes in the field. In agreement with our results, Benelli et al. (2014b) reported that also neem-cake EA and methanol fractions (tested at 100 ppm) exert high effective ovideterrent percentages against *A. albopictus* females, while butanol and the aqueous fractions showed little effectiveness. Interestingly, ovideterrence rates evoked by the NSOs and EAs tested in our experiments overcome that of other plant-borne natural compounds belonging to the same botanical family. For instance, fruit and leaf ethanolic extracts from *Melia azedarach* L. need high dosages to achieve good oviposition deterrence towards *A. aegypti* (e.g. 0.5 g/L of leaf extract and 0.75 g/L of fruit reduce laid eggs to about 30% over the control) (Coria et al. 2008). However, a number of other botanical compounds are also able to strongly deter *A. albopictus* from oviposition (Xue et al. 2001). PON-NEEM [i.e. novel herbal formulation prepared using the oils of *A. indica*, *Pongamia glabra* Vent (Fabaceae)] and their extracts have been proved as a highly effective ovideterrent against *A. albopictus* and *A. aegypti*, also at really low doses (1 ppm) (Maheswaran and Ignacimuthu 2012). Also low concentrations (10 ppm) of acetone fraction of the ethanol extract of *Annona squamosa* L. (Annonaceae) seeds reduce *A. albopictus* laid eggs up to 90% after three days (Kempraj and Bhat 2011). However, the above-mentioned studies (with the exception of Xue et al. 2001) have been conducted through laboratory assays and these results are not fully comparable with the results of our field experiments.

Overall, this is the first report concerning larvicidal toxicity of NSO against *A. albopictus* and ovideterrence of NSO and its fractions against Culicidae in the field. The possibility to use chemicals from NSO and EAs seems very promising since they are effective at lower doses

over synthetic products currently marketed, and can be an advantageous alternative to build newer and safer mosquito control tools.

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Disclosure

The Authors did not have potential conflict of interests relevant to the subject of this research.

References

Abdel-Ghaffar F, Al-Quraishy S, Sobhy H, Semmler M (2008) Neem seed extract shampoo, Wash Away Louse®, an effective plant agent against *Sarcoptes scabiei* mite infesting dogs in Egypt. Parasitol Res 104:145-148

Abdel-Ghaffar F, Semmler M, Al-Rasheid KAS, Mehlhorn H (2009) In vitro efficacy of ByeMite® and Mite-Stop® on developmental stages of the red chicken mite *Dermanyssus gallinae*. Parasitol Res 105:469–471

Abdel-Ghaffar F, Al-Quraishy S, Al-Rasheid KAS, Mehlhorn H (2012) Efficacy of a single treatment of head lice with a neem seed extract: an in vivo and in vitro study on nits and motile stages. Parasitol Res 110:277-280

Al-Quraishy S, Abdel-Ghaffar F, Al-Rasheid KAS, Mehlhorn J, Mehlhorn H (2011) Effects of a neem seed extract (MiteStop®) on mallophages (featherlings) of chicken: in-vivo and in-vitro studies. Parasitol Res 110:617-622

Al-Quraishy S, Abdel-Ghaffar F, Al-Rasheid KAS, Mehlhorn J, Mehlhorn H (2012) Observations on effects of a neem seed extract (MiteStop®) on biting lice (mallophages) and bloodsucking insects parasitizing horses. Parasitol Res 110:335-339

Amer A, Mehlhorn H (2006a) Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae). Parasitol Res 99:466-472

Amer A, Mehlhorn H (2006b) Repellency effect of forty-one essential oils against *Aedes, Anopheles* and *Culex* mosquitoes. Parasitol Res 99:478-490

AOAC (2005) Official Methods of analysis of AOAC International, 18th Edn. AOAC International, Gaithersburg, MD

Batra CP, Mittal PK, Adak T, Sharma VP (1998) Efficacy of neem oil-water emulsion against mosquito immatures. Indian J Malariol 35:15-21

Benedict MQ, Levine RS, Hawley WA, Lounibos LP (2007) Spread of the Tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vect Bor Zoon Dis 7:76-85

Benelli G, Flamini G, Canale A, Cioni PL, Conti B (2012a) Toxicity evaluation of different essential oil formulations against the Mediterranean Fruit Fly *Ceratitis capitata* (Wiedemann) (Diptera Tephritidae). Crop Protect 42:223-229

Benelli G, Flamini G, Canale A, Molfetta I, Cioni PL, Conti B (2012b) Repellence of *Hyptis suaveolens* L. (Lamiaceae) whole essential oil and major constituents against adults of the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Dryophthoridae). Bull Insectol 65:177-183

Benelli G, Canale A, Flamini G, Cioni PL, Demi F, Ceccarini L, Macchia M, Conti B (2013a) Biotoxicity of *Melaleuca alternifolia* (Myrtaceae) essential oil against the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae), and its parasitoid *Psyttalia concolor* (Hymenoptera: Braconidae). Ind Crop Prod 50:596-603

Benelli G, Flamini G, Fiore G, Cioni PL, Conti B (2013b) Larvicidal and repellent activity of the essential oil of *Coriandrum sativum* L. (Apiaceae) fruits against the filariasis vector *Aedes albopictus* Skuse (Diptera: Culicidae). Parasitol Res 112:1155-1161

Benelli G, Canale A, Conti B (2014a) Eco-friendly control strategies against the Asian tiger mosquito, *Aedes albopictus* (Diptera: Culicidae): repellency and toxic activity of plant essential oils and extracts. Pharmacol On Line, 1:44-51

Benelli G, Conti B, Garreffa R, Nicoletti M (2014b) 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

Bowatte G, Perera P, Senevirathne G, Meegaskumbura S, Meegaskumbura M (2013) Tadpoles as dengue mosquito (*Aedes aegypti*) egg predators. Biol Control 67:469-474

Caminade C, Medlock JM, Ducheyne E, McIntryre KM, Leach S, Baylis M, Morse A (2012) Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. J R Soc Interface 9:2708-2717

Canale A, Benelli G, Conti B, Lenzi G, Flamini G, Francini A, Cioni PL (2013) Ingestion toxicity of three Lamiaceae essential oils incorporated in protein baits against the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera Tephritidae). Nat Prod Res 22:2091-2099

Cheah SX, Tay JW, Chan LK, Jaal Z (2013) Larvicidal, oviposition, and ovicidal effects of *Artemisia annua* (Asterales: Asteraceae) against *Aedes aegypti, Anopheles sinensis,* and *Culex quinquefasciatus* (Diptera: Culicidae). Parasitol Res 112:3275-3282

Chinese Pharmacopoeia. (2009) TLC Atlas of Chinese Crude Drugs in Pharmacopoeia of the People's Republic of China Commission. People's Medical Publishing House.

Conti B, Benelli G, Flamini G, Cioni PL, Profeti R, Ceccarini L, Macchia M, Canale A (2012a) Larvicidal and repellent activity of *Hyptis suaveolens* (Lamiaceae) essential oil against the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae). Parasitol Res 110:2013-2021

Conti B, Benelli G, Leonardi M, Afifi UF, Cervelli C, Profeti R, Pistelli L, Canale A (2012b) Repellent effect of *Salvia dorisiana, S. longifolia* and *S. sclarea* (Lamiaceae) essential oils against the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae). Parasitol Res 111:291-299

Conti B, Leonardi M, Pistelli L, Profeti R, Ouerghemmi I, Benelli G (2013) Larvicidal and repellent activity of essential oils from wild and cultivated *Ruta chalepensis* L. (Rutaceae) against

Aedes albopictus Skuse (Diptera: Culicidae), an arbovirus vector. Parasitol Res 112:991-999

Conti B, Flamini G, Cioni PL, Ceccarini L, Macchia M, Benelli G (2014) Mosquitocidal essential oils: are they safe against non-target aquatic organisms? Parasitol Res 113:251-259

Coria C, Almiron W, Valladares G, Carpinella C, Luduena F, Defago M, Palacios S (2008) Larvicide and oviposition deterrent effects of fruit and leaf extracts from *Melia azedarach* L. on *Aedes aegypti* (L.) (Diptera: Culicidae). Bioresour Technol 99:3066-3070

Dua VK, Pandey AC, Raghavendra K, Gupta A, Sharma T, Dash A (2009) Larvicidal activity of neem oil (*Azadirachta indica*) formulation against mosquitoes. Malaria J 8:124

Egho EO (2012) Seeds of neem tree (*Azadirachta indica* A. Juss). Promising biopesticide in the management of cowpea insect pests and grain yield in the early cropping season at Asaba and Abraka, Delta State, Nigeria. J Agric Sci 4:181-189

Elango G, Bagavan A, Kamaraj C, Abduz Zahir A, Abdul Rahuman A (2009) Oviposition-deterrent, ovicidal, and repellent activities of indigenous plant extracts against *Anopheles subpictus* Grassi (Diptera: Culicidae). Parasitol Res (2009) 105:1567–1576

Elango G, Rahuman AA, Kamaraj C, Bagavan A, Zahir AA (2011) Efficacy of medicinal plant extracts against malarial vector, *Anopheles subpictus* Grassi. Parasitol Res 108:1437-1445

Gallo FR, Multari G. Federici E, Palazzino G, Giambenedetti M, Petitto V, Poli F, Nicoletti M. (2011) Chemical fingerprinting of *Equisetum arvense* L. using HPTLC densitometry and HPLC. Nat Prod Res 25:1261-1270

Gallo FR, Multari G, Federici E, Palazzino G, Nicoletti M, Petitto V (2012) The modern analytical determination of Botanicals and similar novel natural products by the HPTLC fingerprint approach. In: Studies in Natural Products Chemistry. Atta-Ur-Rahman (Ed.). Elsevier, 37:217-258

Giatropoulos A, Pitarokili D, Papaioannou F, Papachristos DP, Koliopoulos G, Emmanouel N, Tzakou O, Michaelakis A (2013) Essential oil composition, adult repellency and larvicidal activity of eight Cupressaceae species from Greece against *Aedes albopictus* (Diptera: Culicidae). Parasitol Res 112:1113-23

Gleiser RM, Bonino MA, Zygadlo JA (2011) Repellence of essential oils of aromatic plants growing in Argentina against *Aedes aegypti*. Parasitol Res 108:69-78

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

Govindarajan M, Sivakumar R (2012) Adulticidal and repellent properties of indigenous plant extracts against *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). Parasitol Res 110:1607–1620

Hafeez F, Akram W, Shaalan EA (2011) Mosquito larvicidal activity of citrus limonoids against *Aedes albopictus*. Parasitol Res 109:221-229

Hashmat I, Azad H, Ahmed A (2012) A Nature's drugstore: a review. J. Res J Biol Sci 1:76-79

Hemingway J, Ranson H (2000) Insecticide resistance in insect vectors of human disease. Annu Rev Entomol 45:371-391

Kempraj V, Bhat SK (2011) Acute and reproductive toxicity of *Annona squamosa* to *Aedes albopictus*. Pestic Biochem Physiol 100:82-86

Koliopoulos G, Pitarokili D, Kioulos E, Michaelakis A, Tzakou O (2010) Chemical composition and larvicidal evaluation of *Mentha, Salvia,* and *Melissa* essential oils against the West Nile virus mosquito *Culex pipiens*. Parasitol Res 107:327-335

Koul O, Isman MB, Ketkar CM (1990) Properties and uses of Neem (*Azadirachta indica*) Can J Bot 68:1-11

Kramer WL, Mulla S (1979) Oviposition attractants and repellents of mosquitoes: oviposition responses of *Culex* mosquitoes to organic infusions. Environ Entomol 8:1111-1117

Isman MB (2006) Botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol 51:45-66

Locher N, Al-Rasheid KAS, Abdel-Ghaffar F, Mehlhorn H (2010) In vitro and field studies on the contact and fumigant toxicity of a neem-product (Mite-Stop®) against the developmental stages of the poultry red mite *Dermanyssus gallinae*. Parasitol Res 107:417-423

Maheswaran R, Ignacimuthu S (2012) A novel herbal formulation against dengue vector mosquitoes *Aedes aegypti* and *Aedes albopictus.* Parasitol Res 110:1801-1813

Mehlhorn H, Walldorf V, Abdel-Ghaffar F, Al-Quraishy S, Al-Rasheid KAS, Mehlhorn J (2012) Biting and bloodsucking lice of dogs—treatment by means of a neem seed extract (MiteStop®, Wash Away Dog). Parasitol Res 110:769-773

National Research Council (1992) Neem: a tree for solving global problems. In: Report of an ad hoc panel of the Board on Science and Technology for International Development. National Academy Press, Washington

Nicoletti M, Serafini M, Aliboni A, D'Andrea A, Mariani S (2010) Toxic effects of neem cake extracts on *Aedes albopictus larvae*. Parasitol Res 107:89-84

Nicoletti M (2011) HPTLC fingerprint: a modern approach for the analytical determination of botanicals. Brazil J Pharmacogn 21:818-823

Nicoletti M, Mariani S, Maccioni O, Coccioletti T, Murugan K (2012) Neem cake: chemical composition and larvicidal activity on Asian tiger mosquito. Parasitol Res 111:205-2013

Nicoletti M, Murugan K, Del Serrone P (2014) Current mosquito-borne diseases emergencies in Italy and climate changes. The neem opportunity. Tr Vect Res Parassitol, doi: 10.7243/2054-9881-1-2

Noudjou F, Kouninki H, Ngamo LST, Maponmestsem PM, Ngassoum M, Hance T, Haubruge E, Malaisse F, Marlier M, Lognay GC (2007) Effect of site location and collecting period on the chemical composition of *Hyptis spicigera* Lam. an insecticidal essential oil from North-Cameroon. J Essent Oil Res 19:597-601

Okumu FO, Knols BGJ, Fillinger U (2007) Larvicidal effects of a neem (*Azadirachta indica*) oil formulation on the malaria vector *Anopheles gambiae*. Malaria J 6:63

Panneerselvam C, Murugan K, Kovendan K, Kumar PM (2012) Mosquito larvicidal, pupicidal, adulticidal, and repellent activity of *Artemisia nilagirica* (Family: Compositae) against *Anopheles stephensi* and *Aedes aegypti* Parasitol Res 111:2241–2251

Panneerselvam C, Murugan K (2013) Adulticidal, repellent, and ovicidal properties of indigenous plant extracts against the malarial vector, *Anopheles stephensi* (Diptera: Culicidae) Parasitol Res 112:679-692

Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D (2009) *Aedes albopictus*, an arbovirus vector: from the darkness to light. Microb Infect 11:1177-1185

Perry NB, Anderson RE, Brennan NJ, Douglas MH, Heaney AJ, McGimpsey JA, Smallfield BM (1999) Essential oils from Dalmatian sage (*Salvia officinalis* L.): variations among individuals, plant parts, seasons, and sites. J Agric Food Chem 47:2048-2054

Pushpanathan T, Jebanesan A, Govindarajan M (2006) Larvicidal, ovicidal and repellent activities of *Cymbopogan citrates* Stapf (Graminae) essential oil against the filarial mosquito *Culex quinquefasciatus* (Say) (Diptera: Culicidae). Tropical Biomed 23:208-212

Rajkumar S, Jebanesan A (2005) Repellency of volatile oils from *Moschosma polystachyum* and *Solanum xanthocarpum* against filarial vector *Culex quinquefasciatus* Say. Tropical Biomed 22:139-142

Rajkumar S, Jebanesan A (2009) Larvicidal and oviposition activity of *Cassia obtusifolia* Linn (Family: Leguminosae) leaf extract against malarial vector, *Anopheles stephensi* Liston (Diptera: Culicidae). Parasitol Res 104:337-340

Ram M, Abdin MZ, Khan MA Prabhakar J (2011) HPTLC fingerprint analysis: a quality control for authentication of herbal phytochemicals in high-performance thin-layer chromatography (HPTLC). Springer-Verlag, Berlin

Rao DR, Reuben R, Venugopal MS, Nagasampagi BA, Schmutterer H (1992) Evaluation of neem, *Azadirachta indica*, with and without water management, for the control of culicine mosquito larvae in rice-fields. Med Vet Entomol 6:318-324

Reich E, Schibli A (2007) A high-performance thin-layer chromatography for analysis of medicinal plants. Thieme Medical Publishers, New York

Richter P (2001) Climate changes and mosquito-borne diseases. Environ Health Perspect 109:161-171

Santos-Gomes PC, Fernandes-Ferreira M (2001) Organ- and season-dependent variation in the essential oil composition of *Salvia officinalis* L. cultivated at two different sites. J Agric Food Chem 49:2908-2916

Schmahl G, Al-Rasheid KAS, Abdel-Ghaffar F, Klimpel S, Mehlhorn H (2010) The efficacy of neem seed extracts (Tresan®, MiteStop®) on a broad spectrum of pests and parasites. Parasitol Res 107:261-269

Semmler M, Abdel-Ghaffar F, Al-Rasheid KAS, Mehlhorn H (2010) Nature helps: from research to products against blood sucking parasites. Parasitol Res 105:1483-1487

Sharma VP, Dhiman RC (1993) Neem oil as a sand fly (Diptera: Psychodidae) repellent. J Am Mosq Control Assoc 9:364-366

Su T, Mulla MS (1998a) Antifeedancy of neem products containing Azadirachtin against *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae). J Vector Ecol 23:114-122

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

Tchoumbougang F, Amvam Zollo PH, Fecam Boyom F, Nyegue MA, Bessière JM (2005) Aromatic plants of Tropical Central Africa. XLVIII. Comparative study of the essential oils of four *Hyptis* species from Cameroon: H. lanceolata Poit., H. pectinata (L.) Poit., *H. spicigera* Lam. and *H. suaveolens* Poit. Flavour Fragr J 20:340-343

Xue RD, Barnard DR, Ali A (2001) Laboratory and field evaluation of insect repellents as oviposition deterrents against the mosquito *Aedes albopictus*. Med Vet Entomol 15:126-131

Walldorf V, Mehlhorn H, Al-Quraishy S, Al-Rasheid KAS, Abdel-Ghaffar F, Mehlhorn J (2012) Treatment with a neem seed extract (MiteStop®) of beetle larvae parasitizing the plumage of poultry. Parasitol Res 110:623-627

WHO (2000) General guidelines for methodologies on research and evaluation of traditional medicine. World Health Organization, Geneva

WHO (2009) Guidelines for efficacy testing of mosquito repellents for human skin. WHO/HTM/NTD/WHOPES/2009.4. Control of neglected tropical diseases. World Health Organization, Geneva

Yamany AS, Mehlhorn H, Adham FK (2012) Yolk protein uptake in the oocyte of the Asian tiger mosquito *Aedes albopictus* (Skuse) (Diptera: Culicidae). Parasitol Res 111:1315-1324

Figure 1. HPTLC analysis of neem seed oils (NSOs) of different origins: NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in Bangalore. Mobile phase: toluene:ethyl acetate 7:3 (v/v). Plate visualization: **(a)** at 254 nm; **(b)** at 366 nm after derivatization with anhisaldehyde. Tracks: 1 = NSO1; 2 = NSO2; 3 = NSO3; 4 = salannin.

Figure 2. HPTLC densitometric analysis of tracks from neem seed oils (NSOs) of different origins, NSO1, NSO2, NSO3, and salannin.

Figure 3. Mortality obtained in larvicidal test conducted with neem seed oils and fractions from different production sites against fourth instar larvae of the Asian tiger mosquito, *Aedes albopictus*. NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in Bangalore; EA = ethyl acetate fraction; BU = butanol fraction. Each datum represents the mean of 3 replicates, each one setup with 20 larvae. Means are followed by a standard error. Data followed by the same letters are not statistically different ($P < 0.05$, GLM, Tukey–Kramer HSD test).

Table 1. Toxicity of neem oil and fractions from different production sites against larvae of *Aedes albopictus*. NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in Bangalore; EA = ethyl acetate fraction; BU = butanol fraction; n.d. = not determinate.

Table 2. Relative Median Potency analysis comparing toxicity of neem oil and fractions from different production sites against larvae of *Aedes albopictus*. NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in Bangalore; EA = ethyl acetate fraction; BU = butanol fraction.

Table 3. Oviposition deterrent effect of the neem oils and fractions from different production sites, tested at 200 ppm against *Aedes albopictus* females. Means were followed by standard errors. Each mean value was calculated on three replicates. NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in Bangalore; EA = ethyl acetate fraction; BU = butanol fraction. ER (%) = percent effective repellence. OAI = oviposition activity index. In the ER (%) column, different letters indicate significant differences (GLM, Tukey-Kramer HSD, $P < 0.05$).

Table
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Table 1

Treatment	**LC_{50}** [a]	**95% CI** [b]	**Slope ± SE**	**Intercept ± SE**	***χ^2 (df)*** [c]
NSO1	171.735	160.197-186.797	7.075 ± 1.049	2.643 ± 0.345	**0.02** (1)
NSO1 EA	208.994	185.001-264.684	4.682 ± 0.986	-10.864 ± 2.172	**1.32** (1)
NSO1 BU	287.085	227.282-633.173	4.084 ± 1.199	-10.039 ± 2.655	**1.59** (1)
NSO2	142.286	132.834-152.069	7.482 ± 0.959	-16.109 ± 2.075	**2.06** (1)
NSO2 EA	159.397	150.545-168.891	9.429 ± 1.216	-20.767 ± 2.678	**0.18** (1)
NSO2 BU	171.478	160.783-184.945	7.848 ± 1.116	-17.534 ± 2.463	**1.76** (1)
NSO3	164.140	153.919-176.287	7.775 ± 1.058	-17.224 ± 2.326	**1.95** (1)
NSO3 EA	209.725	169.273-489.615	2.277 ± 0.800	-5.286 ± 1.737	**0.01** (1)
NSO3 BU	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Lethal concentration (LC) killing 50% of exposed larvae. Data are expressed as ppm;

[b] Confidence interval;

[c] Chi-square degrees of freedom;

[d] Values in bold indicate P > 0.05.

Table
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Table 2

Treatment [a]	NSO1	NSO1 EA	NSO1 BU	NSO2	NSO2 EA	NSO2 BU	NSO3
NSO1 EA	0,890 [b]	-	-	-	-	-	-
NSO1 BU	**0,734**	0,824	-	-	-	-	-
NSO2	1,221	**1,372**	**1,664**	-	-	-	-
NSO2 EA	1,076	1,209	**1,467**	0,881	-	-	-
NSO2 BU	0,993	1,116	**1,354**	0,814	0,923	-	-
NSO3	1,043	1,172	**1,422**	0,855	0,970	1,051	-
NSO3 EA	1,025	1,152	**1,398**	0,840	0,953	1,032	0,983

[a] Comparison between treatments (row vs. column) probit analyses of larvicidal activity

[b] Values < 1 indicates that chemical in row is more toxic than chemical in column. Statistically significant values are marked in bold (95% CI ≠ 1).

Table
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Table 3

Compound	Total no. of eggs laid		No. of eggs in bowl		ER (%)	OAI
	Treated	Control	Treated	Control		
NSO1	12	174	4.00 ± 2.08	58.00 ± 9.61	91.64 ± 5.22 ab	-0.871
NSO1 EA	7	154	2.33 ± 1.20	51.33 ± 9.96	94.87 ± 3.24 a	-0.913
NSO1 BU	77	127	25.67 ± 9.87	42.33 ± 13.17	41.54 ± 20.60 bc	-0.245
NSO2	43	134	14.33 ± 10.84	44.67 ± 5.33	70.39 ± 21.20 ab	-0.514
NSO2 EA	10	179	3.33 ± 2.03	59.67 ± 21.21	91.08 ± 5.67 ab	-0.894
NSO2 BU	81	89	27.00 ± 8.74	29.67 ± 9.39	10.35 ± 3.97 c	-0.047
NSO3	27	130	9.00 ± 1.53	43.33 ± 10.87	70.10 ± 3.82 ab	-0.656
NSO3 EA	1	121	0.33 ± 0.33	40.33 ± 8.74	98.55 ± 1.45 a	-0.984
NSO3 BU	111	115	37.00 ± 9.24	38.33 ± 8.95	4.39 ± 2.16 c	-0.018

Figure
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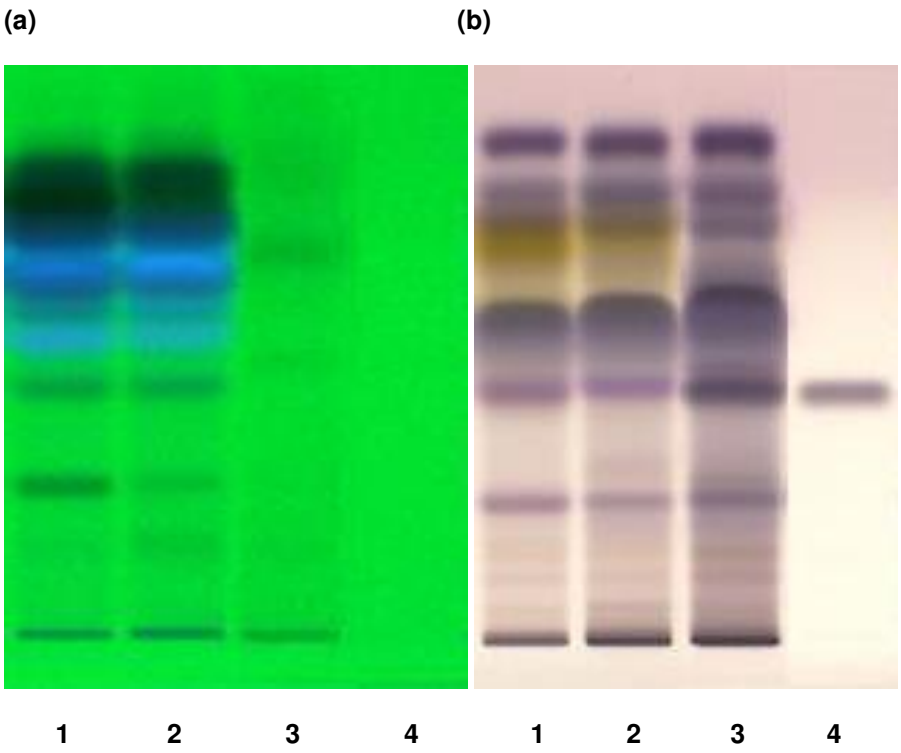
Figure 1



Figure
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Figure 2

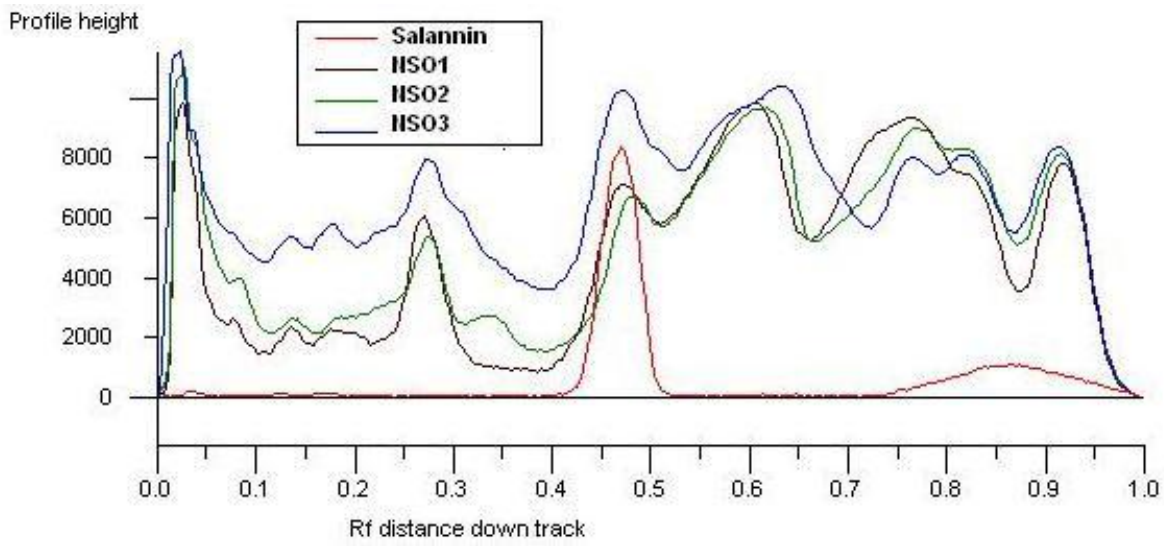

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Figure 3