

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

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Full Title:	Larvicidal and ovideterrent properties of neem oil and fractions against the filariasis vector <i>Aedes albopictus</i> (Diptera: Culicidae): a bioactivity survey across production sites
Article Type:	Original Paper
Abstract:	<p>Neem seed oil (NSO) of <i>Azadirachta indica</i> (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, <i>Aedes albopictus</i>, currently the most invasive mosquito worldwide. Results from bioactivity experiments showed good toxicity of NSOs and EA fractions against <i>A. albopictus</i> fourth instar larvae (with LC50 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 <i>A. albopictus</i> 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 <i>A. albopictus</i> 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.</p>
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1 1 **Larvicidal and ovideterrent properties of neem oil and fractions against the filariasis**
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3 2 **vector *Aedes albopictus* (Diptera: Culicidae): a bioactivity survey across production sites**

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1 20 **Abstract**

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5 22 Neem seed oil (NSO) of *Azadirachta indica* (Meliaceae) contains more than one hundred
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7 23 determined biologically active compounds, and many formulations deriving from them showed
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9 24 toxicity, antifeedancy and repellence against a number of arthropod pests. However, it is widely
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13 26 function of the production site and production process. We used HPTLC (High Performance
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15 27 Thin Layer Chromatography) to investigate differences in chemical constituents of NSOs from
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19 29 diversity among NSOs, with special reference to limonoids. Furthermore, the three NSOs and
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21 30 their fractions of increasing polarities [i.e. ethyl acetate fraction (EA) and butanol fraction (BU)]
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25 32 mosquito, *Aedes albopictus*, currently the most invasive mosquito worldwide. Results from
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27 33 bioactivity experiments showed good toxicity of NSOs and EA fractions against *A. albopictus*
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39 39 effectiveness of BU fractions was found. Concerning ovideterrent activity, no difference due to
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41 40 the production site was found. This is the first report concerning larvicidal toxicity of NSO
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43 41 against *A. albopictus* and ovideterrence against Culicidae in the field. The chance to use
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45 42 chemicals from the NSO EA fraction seems promising, since they are effective at lower doses, if
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47 43 compared to synthetic products currently marketed, and could be an advantageous alternative
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49 44 to build newer and safer mosquito control tools.

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53 46 **Key words:** arbovirus vector; Asian tiger mosquito; botanical by-products; *Azadirachta indica*;
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55 47 HPTLC; Meliaceae; plant-born mosquitocidals

48 Introduction

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50 Since the Middle Ages, plant-borne compounds, such as essential oils and extracts, have
51 been employed for bactericidal, virucidal, fungicidal, parasiticide and insecticidal applications
52 (Amer and Mehlhorn 2006a). After a period of synthetic products dominancy, in the last two
53 decades, renewed efforts have been done to investigate the bioactivity of new plant-borne
54 compounds against an impressive range of arthropod pests, including tephritid flies (Benelli et
55 al. 2012a, 2013a; Canale et al. 2013), foodstuff beetles (Benelli et al. 2012b) and parasites of
56 medical and veterinary importance, with a special focus on mosquitoes (Elango et al. 2011;
57 Conti et al. 2012a; Conti et al. 2013; Giatropoulos et al. 2013; Panneerselvam and Murugan
58 2013; Conti et al. 2014). Many plant essential oils and extracts have been recognised as
59 excellent Culicidae ovicidal (Govindarajan et al. 2011), larvicidal (Amer and Mehlhorn 2006a;
60 Hafeez et al. 2011; Benelli et al. 2013b), adulticidal (Govindarajan et al. 2012; Panneerselvam et
61 al. 2012), growth and/or reproduction inhibitors (Rajkumar and Jebanesan 2005; Pushpanathan
62 et al. 2006), adult repellents (Amer and Mehlhorn 2006b; Koliopoulos et al. 2010; Gleiser et al.
63 2011; Conti et al. 2012b) and oviposition deterrents (Xue et al. 2001; Elango et al. 2009;
64 Rajkumar and Jebanesan 2009). Their use has to face problems of production, formulation,
65 stability and costs. However, natural products still represent one of the most promising
66 possibilities to explore new eco-friendly solutions against mosquitoes.

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

1 77 parasites transmitted by *A. aldopictus*, and vector control remains a pivotal prevention tool.
2
3 78 Although *A. aldopictus* larvae can be killed by organophosphates and insect growth regulators,
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5 79 there is an raising number of resistant mosquito strains ([Hemingway and Ranson 2000](#)).
6
7 80 Biological control tools, based on the release of larvivorous organisms, are frequently not
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9 81 suitable in the majority of urban environments exploited by *A. albopictus* larvae and still require
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11 82 further research ([Bowatte et al. 2013](#)). Therefore, there is an urgent need to find safer and eco-
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13 83 friendly alternatives to enhance the Asian tiger mosquito control strategies.

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15 84 The neem tree, *Azadirachta indica* A. Juss (Meliaceae), is a fast growing evergreen tree
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17 85 native of Indian subcontinent and valued as an important source of eco-friendly phytochemicals
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19 86 for human health and pest management ([National Research Council 1992](#)). The main product of
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21 87 neem is the oil extracted from its seeds. Neem seed oil (NSO) contains at least one hundred
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23 88 biologically active compounds. Among them, major constituents are nor-triterpenes, named
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25 89 limonoids, i.e. azadirachtin, nimbin, nimbidin and nimbolides. NSO is obtained by different
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27 90 extraction methods. Most of the NSO is produced in India by familiar small producers, but many
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29 91 other countries are now producing NSOs. Therefore, considering also the possible different
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31 92 geographical origin of the raw material, combined pre- and post-harvesting factors can result in
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33 93 great differences in constituents present in marketed NSOs, as recently reported by [Gallo et al.](#)
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35 94 ([2012](#)).

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37 95 Many formulations deriving from neem seeds show antifeedancy, fecundity suppression,
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39 96 ovicidal and larvicidal activity, insect growth regulation and/or repellence against insect pests,
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41 97 even at low dosages ([Dua et al. 2009](#); [Egho 2012](#)). For instance, the concentrate extract of
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43 98 neem seeds [e.g. MiteStop, developed by the University spin-off company Alpha-Biocare
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45 99 (Düsseldorf, Germany)] is effective against a number of pests of medical and veterinary
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47 100 importance ([Semmler et al. 2010](#)), including ticks, house dust mites, cockroaches, raptor bugs,
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49 101 cat fleas, bed bugs ([Schmahl et al. 2010](#)), biting and bloodsucking lice ([Al-Quraishy et al. 2011](#);
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51 102 [Al-Quraishy et al. 2012](#); [Abdel-Ghaffar et al. 2012](#); [Mehlhorn et al. 2012](#)), *Sarcoptes scabiei* De
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53 103 Geer mites infesting dogs ([Abdel-Ghaffar et al. 2008](#)), poultry mites ([Abdel-Ghaffar et al. 2009](#);
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55 104 [Locher et al. 2010](#)) and beetle larvae parasitizing the plumage of poultry ([Walldorf et al. 2012](#)).
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57 105 Other advantages arising from the use of neem-based products are no induction of resistance,
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1 106 due to their multiple mode of action against pests and low toxicity rates against vertebrates
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3 107 (Nicoletti et al. 2010, 2012). Overall, the insecticidal properties, environmental safety and public
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5 108 acceptability of neem and its products for the control of insect pests has led to its adoption into
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7 109 some control programs against Diptera pests (Sharma and Dhiman 1993; Su and Mulla 1998a,
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9 110 b), despite some limitations including the relatively high cost of refined products and the low
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11 111 persistence on treated surfaces exposed to sunlight (Isman 2006). Noticeably, emulsified
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13 112 formulations of *A. indica* oil showed an excellent larvicidal potential against different mosquito
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15 113 genera, including *Aedes*, *Anopheles* and *Culex*, also under field conditions (Dua et al. 2009).
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17 114 However, it is widely recognised that botanical products can differ in their chemical composition
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19 115 and bioactivity against a targeted pest as function of the plant's geographical origin and
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21 116 cultivation technique (Tchoumbougang et al. 2005; Noudjou et al. 2007; Conti et al. 2013).
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23 117 Variability in bioactivity has been reported also for neem products (Koul et al. 1990; Gallo et al.
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25 118 2012; Hashmat et al. 2012).

27 119 In this study, we used HPTLC (High Performance Thin Layer Chromatography) to
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29 120 investigate differences in metabolic constituents of three NSOs from different production sites
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31 121 (two from India and one from Thailandia). NSOs and their fractions of increasing polarities [i.e.
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33 122 ethyl acetate fraction (EA) containing the less polar substances, and the butanol fraction (BU),
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35 123 mainly composed by constituents of medium polarity] were then evaluated for their larvicidal
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37 124 toxicity and ovideterrent properties.
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41 126 **Materials and methods**

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43 128 **Neem samples**

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45 130 Samples NSO1 and NSO2 were kindly provided by industrial producers that use them as
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47 131 raw material for preparation of their products. NSO1 was neem seed oil from plants cultivated in
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49 132 India by an industrial producer; NSO2 = was neem seed oil from plants cultivated in Thailandia
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51 133 by an industrial producer. NSO3 was directly collected by one of the Authours (M.N.) from a
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53 134 small local producer in Bangalore (South-East of India). Standards used in the HPTLC analysis
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1 135 were isolated from neem oil and neem cake (i.e. salannin, azadirachtin A, unsaturated and
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3 136 saturated lipids) in previous research (Nicoletti 2011).

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7 138 HPTLC system, materials and sample application

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11 140 HPTLC is the last evolution of planar chromatography (Reich and Schibli 2007; Gallo et al.
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13 141 2012). Allowing to the capacity in evidencing natural products, including also those in very low
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15 142 concentrations, HPTLC is used to perform metabolome studies, like determination of most of
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17 143 the constituents of an extract (Ram et al. 2011; Gallo et al. 2011). Main product of HPTLC
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19 144 analysis is the chromatographic fingerprint, consisting in the individual track typical of the
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21 145 extract or the product. Chromatographic fingerprint analytic approach received important official
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23 146 recognition (WHO 2000; AOAC 2005; Chinese Pharmacopoeia 2009). Plates can be visualized
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25 147 and derivatised in several ways, obtaining multiple information, as well as converted in a series
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27 148 of peaks by densitometric treatment. In such way, the comparison between samples is reliable
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29 149 and facilitated by the visual inspection and samples can be analysed side-by-side and exactly in
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31 150 the same conditions (Gallo et al. 2012). Here, HPTLC was selected to investigate the
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33 151 differences in composition of the tested NSOs and to obtain information about chemical nature
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35 152 of active constituents. Determination of single chemicals (e.g. salannin, azadirachtin A, nimbin)
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37 153 was also achieved by direct comparison with selected standards, obtained in previous research
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39 154 (Nicoletti et al. 2012). Limonoids standards concentration was 2 mM.

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41 155 The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of a Linomat 5 sample
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43 156 applicator using 100 μ L syringes and connected to a nitrogen tank; a chamber ADC 2 containing
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45 157 twin trough chamber 20 x 10 cm; an immersion device III; a TLC Plate Heater III; a TLC
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47 158 visualizer; a TLC scanner 3 linked to win CATS software.

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49 159 Solvents for extraction and HPTLC grade solvents were purchased from Sigma-Aldrich
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51 160 (Milan, Italy) and Carlo Erba (Milan, Italy). Glass plates 20 cm x 10 cm with glass-backed layers
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53 161 silica gel 60 (2 μ m thickness) were from Merck (Darmstadt, Germany). Before use, plates were
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55 162 prewashed with methanol and dried for 3 min at 100 °C. Standards used in HPTLC were
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57 163 isolated in previous researches (Benelli et al. 2014b). Filtered solutions were applied with

1 164 nitrogen flow. The operating conditions were: syringe delivery speed, 10 s μL^{-1} (100 nL s⁻¹);
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3 165 injection volume, 2 μL ; band width, 6 mm; distance from bottom, 15 mm.

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7 167 Development of HPTLC plates

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11 169 The HPTLC plates were developed using the solvent system toluene: ethyl acetate 7:3 (v/v)
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13 170 as mobile phase, in the automatic and reproducibly developing chamber ADC 2, saturated with
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15 171 the same mobile phase for 20 min at room temperature. The developing solvents (i.e. type of
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17 172 solvents and ratios) were carefully optimized before the analyses. The length of the
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19 173 chromatogram run was 80 mm from the point of application. The developed layers were allowed
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21 174 to dry in air for 5 min and then derivatized with a selected solution, including anisaldehyde
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23 175 (1.5 mL *p*-anisaldehyde, 2.5 mL H₂SO₄, 1 mL AcOH in 37 mL EtOH) and/or Natural Product
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25 176 Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate), dried in
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27 177 the open air and then dipped into Macrolog reagent (1 g polyethylene glycol 400 in 20 mL of
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29 178 dichloromethane). Finally, the plates are warmed for 5 min at 120 °C before inspection. All
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31 179 treated plates were then inspected under a UV light at 254 and 366 nm or under reflectance and
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33 180 transmission white light (WRT), respectively, at a Camag TLC visualizer, before and after
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35 181 derivatization. Phenolic nature of compounds at the starting line was confirmed by positive
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37 182 reaction at ferric chloride and ferricyanide tests ([Marini-Bettolo et al. 1981](#); [Graham 1992](#)).

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41 184 Validation, stability and repeatability

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45 186 Band stability and overlapping of bands is a typical analytical challenge for complex
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47 187 mixtures like multi-ingredient products. HPTLC allowed a good separation and visualization of
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49 188 the constituents. Sample solutions were found to be stable at 4 °C for at least 1 month and for at
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51 189 least 3 days on the HPTLC plates.

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53 190 Repeatability was determined by running a minimum of three analyses. R_f values of main
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55 191 selected compounds varied \pm 0.02%. The effects of small changes in the mobile phase
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57 192 composition, mobile phase volume and duration of saturation were minute and reduced by the
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1 193 direct comparison. Results were critically dependent on prewashing of HPTLC plates with
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3 194 methanol ([Benelli et al. 2014b](#)).

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7 196 Fractionation process of NSO samples

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11 198 Fractions of different NSO samples were obtained by repartition of the dry methanol extract
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13 199 of the oil between H₂O and ethyl acetate (1:1, v/v), obtaining the EA together with the aqueous
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15 200 phase. The aqueous phase was retained and partitioned adding an equal volume of *n*-BuOH.
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17 201 Then, BU was obtained ([Benelli et al. 2014b](#)).

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21 203 Larvicidal activity

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25 205 Three groups of twenty fourth-instar larvae were isolated in 250 mL beakers and exposed
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27 206 for 24 hours days to 100, 150 or 200 ppm of the following chemicals: NSOs 1-3, and their
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29 207 respective ethyl acetate and butanol fractions. Each tested product was dissolved in tap water
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31 208 containing 0.1% of Tween 80. Tap water with 0.1% of Tween 80 was used as control. Mortality
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33 209 was checked after 24 h. Larval mortality was reported as an average of three replicates ([WHO](#)
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35 210 [2009](#); [Benelli et al. 2014a](#)). Since no mortality was registered in the control treatment, the
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37 211 mortality percentage rates were not corrected ([Benelli et al. 2013b](#)).

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41 213 Oviposition deterrence in the field

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45 215 Oviposition deterrence of the following chemicals: NSOs 1-3, and their respective ethyl
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47 216 acetate and butanol fractions, was evaluated in the open field. Experiments were carried out in
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49 217 the garden (about 3000 m²) of the entomology laboratory at the Department of Agriculture, Food
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51 218 and Environment, University of Pisa (Italy). All experiments were conducted from June to August
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53 219 2014.

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55 220 Methods described by [Xue et al. \(2001\)](#) and [Benelli et al. \(2014b\)](#) for the oviposition
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57 221 deterrence field tests were followed. Chemicals were tested at the concentration of 200 ppm,
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1 222 dissolved in tap water containing 0.1% of Tween 80. For each treatment, three black plastic
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3 223 containers (10 x 10 x 12 cm) holding 500 mL of water plus the tested compound were placed
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5 224 outdoors. The three containers were arranged in lines and separated by 50 mm each other. A
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7 225 brown masonite ovistrip (200 x 25 mm) was placed in each of the three containers.

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9 226 For each test, a control treatment was run placing a group of control containers in proximity
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11 227 (about 80 cm) of the group of treated oviposition containers. All control treatments contained tap
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13 228 water with 0.1% of Tween 80. Both for treatments and controls, the positions of the container
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15 229 were alternated between the different replicates, to avoid oviposition bias due to positional
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17 230 effects ([Benelli et al. 2014b](#)).

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19 231 Masonite ovistrips were checked daily. After seven days, they were removed, air dried in
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21 232 the laboratory and the number of *A. albopictus* eggs laid in treated and control ovistrips was
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23 233 counted using a stereomicroscope (Leica, Germany). The percent effective repellence for each
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25 234 concentration was calculated using the following formula ([Rajkumar and Jebanesan 2009](#);
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27 235 [Benelli et al. 2014b](#)):

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

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35 239 Oviposition activity index (OAI) was calculated using the formula ([Cheah et al. 2013](#); [Benelli](#)
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37 240 [et al. 2014b](#)):

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

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45 244 NT = total number of eggs in the test solution and NC = total number of eggs in the control
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47 245 solution.

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49 246 Oviposition active index of +0.3 and above are considered as attractants while those with
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51 247 -0.3 and below are considered as repellents ([Kramer and Mulla 1979](#)). Positive values indicate
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53 248 that more eggs were deposited in the test containers than in the control containers and that the
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55 249 test solutions were attractive. Conversely, negative values indicate that more eggs were
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57 250 deposited in the control containers than in the test containers and that the test solutions were a

1 251 deterrent (Cheah et al. 2013; Benelli et al. 2014b).

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5 253 Data analysis

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9 255 Larval mortality data were transformed into arcsine√proportion values before statistical
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11 256 analysis. Data were processed with JMP, using a General Linear Model (GLM) with three
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13 257 factors, the neem geographical origin, the tested chemical (oil, ethyl acetate fraction and
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15 258 butanol fraction) and the dosage (100, 150 or 200 ppm): $y_j = \mu + O_j + C_j + D_j + O_j*C_j + O_j*D_j +$
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17 259 $C_j*D_j + O_j*C_j*D_j + e_j$, in which y_j is the observation, μ is the overall mean, O_j the origin ($j = 1-3$),
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19 260 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
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21 261 interaction origin*dosage, C_j*D_j the interaction chemical*dosage, $O_j*C_j*D_j$ the interaction
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23 262 origin*chemical*dosage, and e_j the residual error. Averages were separated by Tukey-Kramer
24
25 263 HSD test. $P < 0.05$ was used for the significance of differences between means.

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27 264 Median lethal dose (LD_{50}) against Asian tiger mosquito larvae was calculated by Log-probit
28
29 265 regressions by the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Significant differences
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31 266 between LD_{50} values were determined by estimation of confidence intervals of the relative
32
33 267 median potency. Differences among LD_{50} values were judged as statistically significant when
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35 268 values in the 95% confidence interval of relative median potency analyses were $\neq 1.0$.

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37 269 Effective oviposition deterrence percentage data were transformed into arcsine√proportion
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39 270 values, before statistical analysis. Effective oviposition deterrence percentage data were
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41 271 processed with JMP, using the above-described GLM with two factors (the neem origin and the
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43 272 tested chemical) and their interaction. Averages were separated by Tukey-Kramer HSD test. P
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45 273 < 0.05 was used for the significance of differences between means.

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48 49 275 **Results**

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53 277 HPTLC and densitometric analyses

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57 279 Compositions of NSOs from different production sites were compared. Metabolites spots,

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1 280 diffused in the tracks according to the polarity of constituents, showed similarities of fingerprints
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3 281 NSO 1 and 2 (**Figure 1**). Identification of raw material was assured by the presence of salannin
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5 282 (Rf = 0.42), a typical maker of NSO. In comparison with the spot of azadirachtin A (Rf = 0.23),
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7 283 salannin appeared the main limonoid spot. Spots concerning lipids were present at Rf values, at
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9 284 ca. 0.80, due to unsaturated fatty acids and alcohols, and at Rf ca. 0.50, due to saturated and
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11 285 unsaturated triglycerides (based on NMR data, dataset available under request). The most
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13 286 interesting feature of the plates was the presence of compounds with high fluorescent reaction
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15 287 at between Rf 0.55-0.66, that were visible in NSO samples at 366 nm, both before and after
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17 288 derivatization (**Figures 1 and 2**).

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21 290 Larvicidal activity

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25 292 NSOs exhibited dose-dependent toxic activity against *A. albopictus* larvae (**Figure 3**). We
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27 293 found a significant effect of the production site ($F = 21.342$; $d.f. = 2$; $P < 0.001$), tested chemical
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29 294 ($F = 60.854$; $d.f. = 2$; $P < 0.001$), dose ($F = 157.478$; $d.f. = 2$; $P < 0.001$), and the interactions
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31 295 production site*chemical ($F = 13.951$; $d.f. = 4$; $P < 0.001$), chemical *dose ($F = 6.501$; $d.f. = 4$; P
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33 296 < 0.001), production site*dose ($F = 14.271$; $d.f. = 4$; $P < 0.001$), while the interaction production
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35 297 site*chemical*dose was not significant ($F = 1.578$; $d.f. = 8$; $P = 0.153$).

36
37 298 LC₅₀ values were similar among the tested NSOs, as well as among EAs (**Table 1**).
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39 299 However, this was not true for BUs, where LC₅₀ showed significant differences according to
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41 300 different producers (**Table 2**, see also **Figure 3**).

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45 302 Oviposition deterrence

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49 304 NSOs tested at 200 ppm were able to deter oviposition in *A. albopictus* (**Table 3**). Results
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51 305 showed a significant effect of tested chemical ($F = 41.750$; $d.f. = 2$; $P < 0.001$), while the effects
52
53 306 of production site ($F = 2.345$; $d.f. = 2$; $P < 0.125$) and the interaction production site*chemical
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55 307 were not significant ($F = 1.043$; $d.f. = 4$; $P < 0.413$).

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57 308 ER and OAI showed comparable efficacy of NSO1 (ER = 91.64 ± 5.22; OAI = -0.871),
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1 309 NSO2 (ER = 70.39 ± 21.20; OAI = -0.514) and NSO3 (ER = 70.10 ± 3.82; OAI = -0.656). Also
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3 310 EA fractions showed comparable ER (94.87 ± 3.24 vs. 91.08 ± 5.67 vs. 98.55 ± 1.45,
4
5 311 respectively) and OAI (-0.871 vs. -0.894 vs. -0.984) values (**Table 3**). By contrast, BU fractions
6
7 312 were less effective in inducing ovideterrence in *A. albopictus* females, as observed in BU
8
9 313 fraction of NSO1 (ER = 41.54 ± 20.60; OAI = -0.245), BU fraction of NSO2 (10.35 ± 3.97; OAI =
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11 314 -0.047) and BU fraction of NSO3 (ER = 4.39 ± 2.16; OAI = -0.018) (**Table 3**).

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14 15 316 **Discussion**

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19 318 HPTLC analyses highlighted that a number of spots in fingerprint tracks were attributed to
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21 319 nor-triterpenoids and fatty constituents, while several others were related to high conjugated
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23 320 unsaturated aromatic structures, actually under study. Their structures are really different from
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25 321 those of limonoids, which so far have been considered as responsible of bioactivity ([Nicoletti et
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27 322 al. 2012](#)). Results from bioactivity experiments showed that NSOs and its fractions were able to
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29 323 exert significant toxicity against *A. albopictus* fourth instar larvae, and the effect was dose-
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31 324 dependent. Previous researches highlighted the toxicity of neem oil against larvae of several
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33 325 Culicidae species. For instance, application of 5% neem oil-water emulsion at 50 mL/m² in pools
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35 326 lead to 100% and 51.6% reduction of III-IV instar larvae of *Anopheles stephensi* Liston and
36
37 327 *Culex quinquefasciatus* Say after 24 h. Moreover, application of 10% emulsion in desert coolers
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39 328 against *Aedes aegypti* (L.) at dosages ranging from 40 to 80 mL/cooler resulted in complete
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41 329 inhibition of pupal production ([Batra et al. 1998](#)). NSO from seeds cultivated in costal Kenya
42
43 330 showed very good larvicidal properties against *Anopheles gambiae* Giles (LC₅₀ = 11 ppm)
44
45 331 ([Okumu et al. 2007](#)), while LC₅₀ of an industrial neem oil formulation (i.e. neem oil coformulated
46
47 332 with polyoxyethylene ether, sorbitan dioleate and epichlorohydrin) against *A. stephensi*, *C.*
48
49 333 *quinquefasciatus* and *A. aegypti* were 1.6, 1.8 and 1.7 ppm respectively ([Dua et al. 2009](#)). Our
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51 334 data extend this survey to *A. albopictus*, showing good larvicidal activity of NSOs and EAs
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53 335 against *A. albopictus* fourth instar larvae (with LC₅₀ values ranging from 142.28 to 209.73 ppm).
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55 336 The production site has noticeable impact on NSO toxicity against insect pests, as already
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57 337 found for other plant species ([Perry et al. 1999](#); [Santos-Gomes and Fernandes-Ferreira 2001](#);
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1 338 Tchoumbougang et al. 2005; Noudjou et al. 2007; see also Conti et al. 2013). Biototoxicity of
2
3 339 NSOs against mosquitoes seems mainly due to constituents of EAs, while BUs showed little or
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5 340 no effectiveness against *A. albopictus* larvae. Lastly, toxicity against larvae of *A. albopictus* has
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7 341 been recently validated also testing neem-cake, a by-product of NSO extraction, still rich in
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9 342 limonoids (Rao et al. 1992; Nicoletti et al. 2010; Benelli et al. 2014a).

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11 343 All tested NSOs, as well as their EAs, were able to deter oviposition of *A. albopictus*
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13 344 females in the field, while the scarcity of chemicals in NSO BU fractions lead to poor efficacy.
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15 345 Compounds tested in our experiments were effective at low dosages (200 ppm). This is the first
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17 346 report about ovideterrent activity of neem oil against mosquitoes in the field. In agreement with
18
19 347 our results, Benelli et al. (2014b) reported that also neem-cake EA and methanol fractions
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21 348 (tested at 100 ppm) exert high effective ovideterrent percentages against *A. albopictus* females,
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23 349 while butanol and the aqueous fractions showed little effectiveness. Interestingly, ovideterrence
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25 350 rates evoked by the NSOs and EAs tested in our experiments overcome that of other plant-
26
27 351 borne natural compounds belonging to the same botanical family. For instance, fruit and leaf
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29 352 ethanolic extracts from *Melia azedarach* L. need high dosages to achieve good oviposition
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31 353 deterrence towards *A. aegypti* (e.g. 0.5 g/L of leaf extract and 0.75 g/L of fruit reduce laid eggs
32
33 354 to about 30% over the control) (Coria et al. 2008). However, a number of other botanical
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35 355 compounds are also able to strongly deter *A. albopictus* from oviposition (Xue et al. 2001).
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37 356 PON-NEEM [i.e. novel herbal formulation prepared using the oils of *A. indica*, *Pongamia glabra*
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39 357 Vent (Fabaceae)] and their extracts have been proved as a highly effective ovideterrent against
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41 358 *A. albopictus* and *A. aegypti*, also at really low doses (1 ppm) (Maheswaran and Ignacimuthu
42
43 359 2012). Also low concentrations (10 ppm) of acetone fraction of the ethanol extract of *Annona*
44
45 360 *squamosa* L. (Annonaceae) seeds reduce *A. albopictus* laid eggs up to 90% after three days
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47 361 (Kemprij and Bhat 2011). However, the above-mentioned studies (with the exception of Xue et
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49 362 al. 2001) have been conducted through laboratory assays and these results are not fully
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51 363 comparable with the results of our field experiments.

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53 364 Overall, this is the first report concerning larvicidal toxicity of NSO against *A. albopictus* and
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55 365 ovideterrence of NSO and its fractions against Culicidae in the field. The possibility to use
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57 366 chemicals from NSO and EAs seems very promising since they are effective at lower doses
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1 367 over synthetic products currently marketed, and can be an advantageous alternative to build
2
3 368 newer and safer mosquito control tools.

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6
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10
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14
15 374 chemical characterization of neem oils and fractions. The mention of commercial neem-based
16
17 375 products did not constitute and endorsement by the authors and their institutions.

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20
21 377 **Disclosure**

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24
25 379 The Authors did not have potential conflict of interests relevant to the subject of this
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27 380 research.

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1 594 **Figure 1.** HPTLC analysis of neem seed oils (NSOs) of different origins: NSO1 = neem seed oil
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3 595 from an industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer;
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5 596 NSO3 = neem seed oil from a small Indian producer in Bangalore. Mobile phase: toluene:ethyl
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7 597 acetate 7:3 (v/v). Plate visualization: **(a)** at 254 nm; **(b)** at 366 nm after derivatization with
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9 598 anhisaldehyde. Tracks: 1 = NSO1; 2 = NSO2; 3 = NSO3; 4 = salannin.

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13 600 **Figure 2.** HPTLC densitometric analysis of tracks from neem seed oils (NSOs) of different
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15 601 origins, NSO1, NSO2, NSO3, and salannin.

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19 603 **Figure 3.** Mortality obtained in larvicidal test conducted with neem seed oils and fractions from
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21 604 different production sites against fourth instar larvae of the Asian tiger mosquito, *Aedes*
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23 605 *albopictus*. NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem seed oil
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25 606 from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in
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27 607 Bangalore; EA = ethyl acetate fraction; BU = butanol fraction. Each datum represents the mean
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29 608 of 3 replicates, each one setup with 20 larvae. Means are followed by a standard error. Data
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31 609 followed by the same letters are not statistically different ($P < 0.05$, GLM, Tukey–Kramer HSD
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33 610 test).

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35 611 **Table 1.** Toxicity of neem oil and fractions from different production sites against larvae of
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37 612 *Aedes albopictus*. NSO1 = neem seed oil from an industrial Indian producer; NSO2 = neem
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39 613 seed oil from an industrial Thai producer; NSO3 = neem seed oil from a small Indian producer in
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41 614 Bangalore; EA = ethyl acetate fraction; BU = butanol fraction; n.d. = not determinate.

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45 616 **Table 2.** Relative Median Potency analysis comparing toxicity of neem oil and fractions from
46
47 617 different production sites against larvae of *Aedes albopictus*. NSO1 = neem seed oil from an
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49 618 industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 =
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51 619 neem seed oil from a small Indian producer in Bangalore; EA = ethyl acetate fraction; BU =
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53 620 butanol fraction.

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1 622
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3 623 **Table 3.** Oviposition deterrent effect of the neem oils and fractions from different production
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5 624 sites, tested at 200 ppm against *Aedes albopictus* females. Means were followed by standard
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7 625 errors. Each mean value was calculated on three replicates. NSO1 = neem seed oil from an
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9 626 industrial Indian producer; NSO2 = neem seed oil from an industrial Thai producer; NSO3 =
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11 627 neem seed oil from a small Indian producer in Bangalore; EA = ethyl acetate fraction; BU =
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13 628 butanol fraction. ER (%) = percent effective repellence. OAI = oviposition activity index. In the
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15 629 ER (%) column, different letters indicate significant differences (GLM, Tukey-Kramer HSD, P <
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17 630 0.05).
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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 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

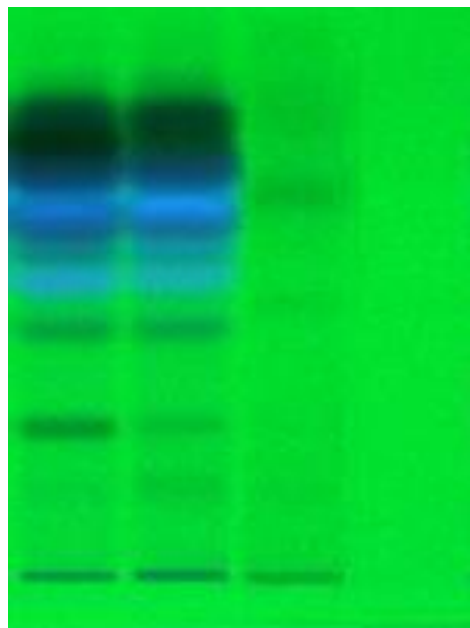
^b Values < 1 indicates that chemical in row is more toxic than chemical in column. Statistically significant values are marked in bold (95% CI ≠ 1).

Table 3

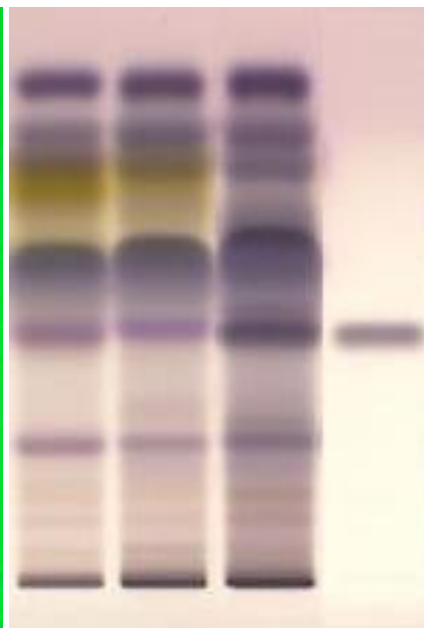
Compound	Total no. of eggs laid		No. of eggs in bowl		ER (%)	OAI
	Treated	Control	Treated	Control		
NSO1	12	174	4.00 ± 2.08	58.00 ± 9.61	91.64 ± 5.22 ab	-0.871
NSO1 EA	7	154	2.33 ± 1.20	51.33 ± 9.96	94.87 ± 3.24 a	-0.913
NSO1 BU	77	127	25.67 ± 9.87	42.33 ± 13.17	41.54 ± 20.60 bc	-0.245
NSO2	43	134	14.33 ± 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 1

(a)



(b)



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Figure 2

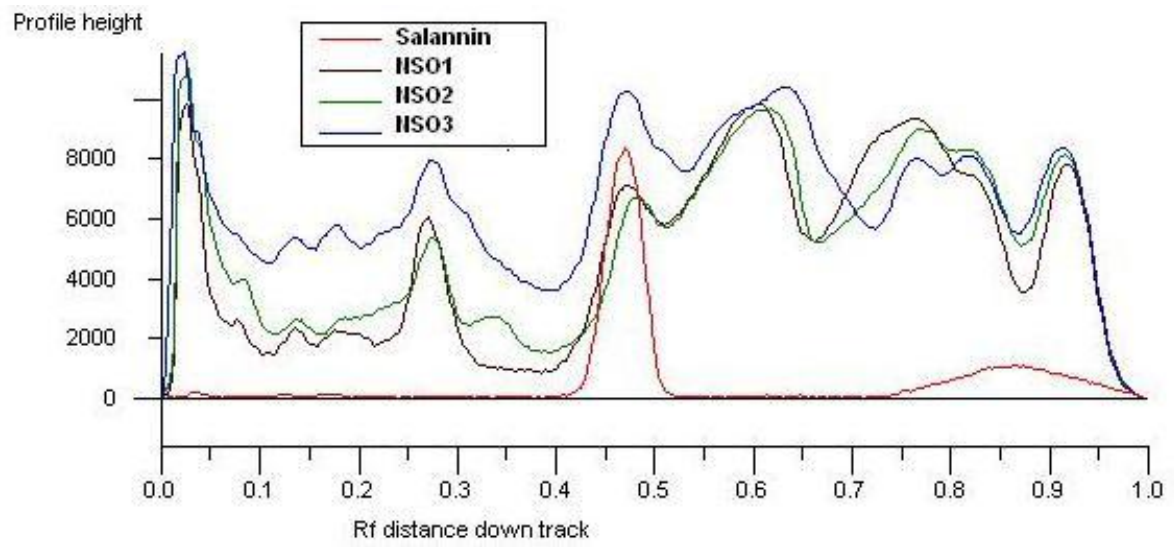


Figure 3

