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The recent outbreaks of Zika virus: Mosquito control faces a further challenge

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ABSTRACT

The recent outbreaks of Zika virus infection occurring in South America, Central America and the Caribbean, represent the most recent of four key arrivals of arboviruses in the Western Hemisphere over the last 20 years. Zika virus is mainly vectored by *Aedes* mosquitoes. The development of effective and eco-friendly mosquito control methods is required in order to minimize the negative effects of currently marketed synthetic pesticides, including multidrug resistance. In this scenario, natural product research can afford solutions as part of integrated pest management strategies. In this review, we focused on neem (*Azadirachta indica*) products as sources of cheap control tools of *Aedes* vectors. Current knowledge on the larvicidal, pupicidal, adulticidal and oviposition deterrent potential of neem-borne products against the arbovirus vectors *Aedes aegypti* and *Aedes albopictus* is reviewed. Furthermore, we considered the rising importance of neem extraction by-products as sources of bio-reducing agents for the synthesis of nanoformulated mosquitocides. The last section examined biosafety and non-target effects on neem-borne mosquitocides in the aquatic environment. Overall, we support the employ of neem-borne molecules as an advantageous alternative to build newer and safer *Aedes* control tools, in the framework of Zika virus outbreak prevention.

1. Introduction

Arthropods are dangerous vectors of deadly parasites and pathogens, which may hit as epidemics or pandemics in the increasing world population of humans and animals[1,2]. The scenario of arthropod-borne diseases, due to the spread of highly infective viruses or other microorganisms by arthropod vectors, is rapidly changing. Currently, they are far to be effectively controlled, and millions of humans and animals are yearly subjected to malaria, yellow fever, dengue, West Nile, chikungunya and filariasis[3,4].

The globalization process and climate changing often play a key role in the spreading of arbovirus infections. For instance, the emergence of blue tongue virus (BTV) in Europe is a noteworthy study case[5,6]. BTV is a devastating disease of ruminants causing more than one million of sheep deaths[7], with epidemic spread in the Mediterranean area and in particular in Sardinia island[8,9]. BTV replicates in different ruminant species, but the severe disease is mostly restricted to certain breeds of sheep, producing fine wool, with annual losses of US$ million[7]. BTV is transmitted among ruminants by *Culicoides* biting midges (Diptera: Ceratopogonidae[10,11]. BTV has historically made only brief and sporadic incursion in Europe, until 1998, when six strains of BTV have spread across 12 countries and 800 km, reaching the north of Europe, including UK and Scandinavia. Until 1997, the limit of *Culicoides imicola* was stable in the ordinary limit including Portugal, Southwestern Spain and some Greek islands[5]. From 1998, BTV epidemic began, following two routes: the first from Greek islands, close to the Turkish coast, spread to West and later to North, involving Bulgaria, Kosovo, Albania, Macedonia, Serbia, Montenegro and Croatia. The second route mainly occurred in 2000, spreading from Tunisia and Algeria into Sardinia, Sicilia and other

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parts of Italy[10]. The hypothesis is that BTV was so far restricted to tropical and subtropical areas worldwide. Its entrance in Europe was probably due to the presence of disease-resistant host animals or by the dispersal of infected *Culicoides* midges, but transmission in Europe is considered a consequence of the climatic changes, being the BTV-*Culicoides* system exquisitely sensible to changes in climate. Notably, in Europe climate changes generated higher temperatures during the cold season, and fewer frost days during the winter. The BTV emergency in Europe was coincident with a period of warming (1976–2000). During incursions, only one or two countries were affected at a time and only a single BTV serotype was involved. Once the invasion reached the new habitat, its eradication is difficult to reach[11]. The migrations and evolution of serotypes, including the emergence of dangerous pathogen and parasite strains, were carefully monitored by the scientific community. Often, despite extensive advises from the scientific side, any invasion finds local authorities largely unprepared and first measures have to wait long periods. The story of the recent explosive epidemic spread of Zika virus is similar to BTV and other vector-borne diseases, such as the recent olive quick decline syndrome. In four years, the olive quick decline syndrome caused the death of thousands of olive trees in the South Italy and was menacing the survival of olive grown in all the Mediterranean area. The pathogenic agent, the bacterium *Xylella fastidiosa* (*X. fastidiosa*), was known from hundreds of years in America and at least from decades in Southern Italy, without any devastating effects[11].

Zika was discovered in Uganda in 1947 during mosquito and primate surveillance, and remained for long time an obscure virus confined to a narrow equatorial belt, running across Africa and into Asia. In that period, Zika was predominantly confined in wild primates and arboreal mosquitoes, such as *Aedes africanus*. Rarely, it caused recognized "spillover" infections in humans, even in highly enzootic areas. A peculiar and remarkable aspect of the emergence of Zika virus infection is the tendency to follow *Aedes*-transmitted epizootics and epidemics, as highlighted for the United States current scenario. Zika is the most recent of four unexpected arrivals of important arthropod-borne viral diseases in the Western Hemisphere over the past 20 years. It followed dengue, which entered this hemisphere stealthily over decades and then more aggressively in the 1990s. Later, West Nile virus emerged in 1999, and chikungunya emerged in 2013. An analogous pattern began in 2013, when chikungunya spread pandemically from west to east, and Zika later followed. Zika has now circled the globe, arriving not only in the Americas but also in the country of Cape Verde in West Africa, near its presumed ancient ancestral home[2,12].

Unfortunately, as already known for other arboviruses such as dengue, West Nile and chikungunya, no vaccines or other specific treatments are available for Zika virus infection, and avoidance of mosquito bites remains the best strategy[13]. Besides territorial control, the development of vaccines and further research on Zika virus potential complications, the attention of researches focused on developing solutions for effective control of *Aedes* vectors (Figure 1). Behaviour-based control tools and the sterile insect technique recently received renewed attention[3,4,14]. However, current mosquito control in tropical and subtropical areas worldwide is still based on the application of mosquito ovicides, larvicides, pupicides and adulticides, as well as the employ of repellents applied on bed nets and uncovered body parts[2].

Synthetic insecticides are often harmful for human health and the environment, and lead to the development of resistance in the targeted pest populations[15,16]. Therefore, it may be helpful to consider natural products as suitable sources of eco-friendly mosquitocides[11,17], as key part in control of pests. Among the natural insecticides, a prominent place can be assigned to the neem [*Azadirachta indica* (*A. indica*)] seed oil[18], corresponding to several of the characters before reported. However, the cost of neem oil is actually quite high, limiting its use on large scale. A solution could be the use of neem cake, which still contains most of the activity of the neem oil, but is a cheap by-product[19,20], being obtained as waste during the neem kernels expression. Furthermore, a key problem in the utilization of neem-borne products is the variability among production sites and the rapid degradation and loss of efficacy in the field, due to photodecomposition of limonoids azadirachtin, the main active constituents[21]. The first problem could be solved with the utilization of high performance thin-layer chromatography, while the second may benefit by the employ of green nanotechnologies[11,22-25].

The development of effective and eco-friendly Culicidae control methods is required in order to minimize negative effects of currently marketed synthetic pesticides. In this scenario, natural product research can afford solutions as part of integrated pest management strategies. Here, we focused on neem products as sources of cheap control tools of *Aedes* vectors. Current knowledge on the larvicidal, pupicidal, adulticidal and oviposition deterrent potential of neem-borne products against the arbovirus vectors *Aedes aegypti* (*Ae. aegypti*) and *Aedes albopictus* (*Ae. albopictus*) is reviewed. Furthermore, we considered the rising importance of neem extraction by-products as sources of bio-reducing agents for the synthesis of nanoformulated mosquitocides. The last section deals with biosafety and non-target effects on neem-borne mosquitocides in the aquatic environment.

2. Neem-borne compounds as eco-friendly tools against *Aedes* mosquitoes

The neem insecticidal activity has been reported in about one hundred published researches (*e.g.*[26-30]; see[31] for a recent review), reporting insecticidal activity against more than 400 species. The neemome is particularly complex, with more than 100 biologically active compounds, and many formulations deriving from them showed antifeedancy, fecundity suppression, ovicidal and larvicidal activity, insect growth regulation and/or repellence against a wide range of arthropod pests of public health importance including ticks, house dust mites, cockroaches, raptor bugs, cat fleas, bed bugs, biting and bloodsucking lice, *Sarcoptes scabiei* mites infesting dogs, poultry mites, beetle larvae parasitizing the plumage of poultry and sand flies[30,32-40], as well as mosquito vectors[19,31,41-45].

Neem oil is toxic towards the larvae of several Culicidae species, including the Zika vectors belonging to the genus *Aedes*. For instance, applications of 10% emulsion in desert coolers against *Ae. aegypti* at dosages ranging from 40 to 80 mL/cooler resulted in complete inhibition of pupal production[46]. The application of 5% neem oil-water emulsion at 50 mL/m^2 in pools leads to 100% and 51.6% reduction of third and fourth instar larvae of *Anopheles stephensi* (*An. stephensi*) and *Culex quinquefasciatus* (*Cx. quinquefasciatus*), after 24 h[46]. The LC_{50} of neem oil co-formulated with polyoxyethylene ether, sorbitan dioleate and epichlorohydrin against *Ae. aegypti* larvae was 1.7 ppm, and similar results were obtained also against malaria vectors (*An. stephensi*, LC_{50} = 1.6 ppm) and filariasis vectors (*Cx. quinquefasciatus*, LC_{50} = 1.8 ppm[47],

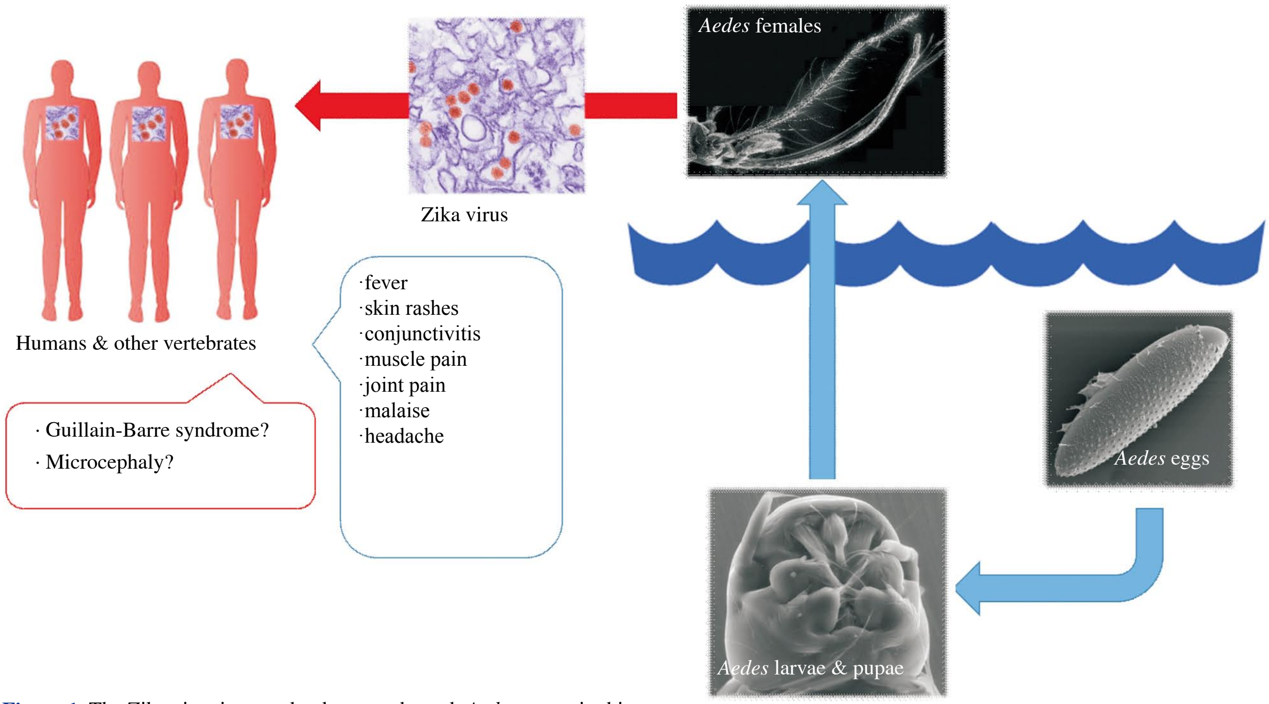

Figure 1. The Zika virus is spread to humans through *Aedes* mosquito bites.
Aedes young instars live in aquatic environments, including water reservoirs in urban and peri-urban areas. *Aedes* females vectored the Zika virus during blood feeding. Zika virus symptoms often include fever, rash, joint pain, and conjunctivitis. The illness is usually mild with symptoms lasting from several days to a week. Severe disease requiring hospitalization is uncommon. On the red arrow (indicating blood feeding), a digitally colorized transmission electron micrograph of Zika virus (Flaviviridae). The virus particles (colored in red), are 40 nm in diameter, with an outer envelope, and an inner dense core (photo credit: TEM micrograph of Zika virus was kindly provided by Dr. C. Goldsmith, Public Health Image Library, Centers for Disease Prevention and Control; SEM micrographs of *Aedes* mosquitoes: Dr. R. Antonelli).

while neem oil from seeds cultivated in costal Kenya showed larvicidal potential against *Anopheles gambiae* (LC_{50} = 11 ppm)[48]. More recently, the neem seed oils from different production sites were tested against the larvae of *Aedes albopictus*. The oils and their ethyl acetate fractions showed good larvicidal activity towards fourth instar larvae (LC_{50} values ranging from 142.28 to 209.73 ppm[19]. As regards to oviposition deterrence, a good example is PONNEEM, a novel herbal formulation prepared using the oils of *A. indica* and *Pongamia glabra* (Fabaceae), which has been proven as a highly effective ovideterrent against *Ae. aegypti* and *Ae. albopictus*, at really low doses (1 ppm)[49].

3. Neem byproducts: potential against *Aedes* vectors

Neem cake is an important source of compounds effective against different mosquito vectors of public health importance. The majority of available information focuses on *Ae. albopictus* and *Culex* species as study targets[4], while moderate information is available about *Ae. Aegypti*. Shanmugasundaram *et al.*[50] tested neem cake against fourth-instar larvae of *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi* and reported good toxic properties [LC_{50} = 0.29% (w/v), 0.56%, and 0.45%, respectively]. Rao *et al.*[51] showed that neem cake powder applied in rice fields at a dose of 500 kg/ha, either alone or coated over urea, has been able to exert a strong reduction in the abundance of *Cx. quinquefasciatus* late-instar larvae and pupae[51]. Nicoletti *et al.*[52] studied the bioactivity of neem cake fractions of increasing polarity (50 ppm) against eggs of *Ae. albopictus*, showing no differences in egg hatching over control. When newly emerged larvae were allowed to develop in the neem cake solutions, higher mortality rates have been reported after 8 days for the hexane and ethyl acetate fractions, over the butanol fraction, aqueous fraction and control. The neem cake methanol extract was able to block surviving *Ae. albopictus* individuals at larval stages[52,53]. Later on, Nicoletti *et al.*[54] extended the neem cake bioactivity survey to *Ae. albopictus*, testing six commercial samples. Notably, three samples did not show significant mosquitocidal activity on newly hatched larvae, while two of them were not toxic towards late-instar larvae[54]. This highlights the key importance of comparative approaches in bioactivity surveys testing raw products from different production processes against arthropods of medical importance[31]. In addition, Benelli *et al.*[55] did not find consistent differences among neem cake methanol, ethyl acetate, butanol and aqueous fractions, since all of them killed more than 80% of *Ae. albopictus* larvae after 15 days. It has been formulated that the differences in larvicidal activity of neem cake products can be partially due to the amounts of minor constituents synergizing the insecticidal effect of major constituents[4,55]. The role of constituents, previously considered as secondary, is now becoming increasingly important, as evidenced by the *Cannabis sativa* recent case[56].

As regards to the ovideterrent properties of neem cake against Culicidae, recently, neem cake fractions of increasing polarity have been tested in field experiments as oviposition deterrents against females of *Ae. albopictus*[29]. The neem cake *n*-hexane, methanol and ethyl acetate fractions were able to exert effective repellence percentages over the control, even if tested at low dosages (100 ppm: 71.33%, 88.59% and 73.49% of effective repellence, respectively). Conversely, the *n*-butanol and the aqueous fractions have shown little oviposition effective repellence rates against *Ae. albopictus* (100 ppm: 22.72% and 17.06% of effective repellence, respectively). The highest oviposition activity index was achieved by the *n*-hexane fraction (–0.82), followed by the ethyl acetate fraction (–0.63) and the methanol fraction (–0.62). A lower oviposition activity index was achieved by the *n*-butanol fraction (–0.14) and the aqueous fraction (–0.09)[29].

Interestingly, the oviposition deterrence exerted by the neem cake *n*-hexane, methanol and ethyl acetate fractions overcomes that of other plant-borne natural compounds belonging to the same botanical family. For instance, in laboratory conditions, the fruit and leaf ethanol extracts from *Melia azedarach* needed high dosages to achieve substantial oviposition deterrence towards *Ae. aegypti* (*e.g.* 0.5 g/L of leaf extract and 0.75 g/L of fruit extract evoke a reduction of laid eggs to about 30% if compared to the control)[57].

4. Neem-based nanosynthesis of mosquitocides

Nanoparticles are defined as particles containing active compounds, for their increase of activity or delivery or protection. The plant-mediated synthesis of metal nanoparticles (also known as "green synthesis") is advantageous over chemical and physical methods, since it is cheap, single-step, and does not require high pressure, energy, temperature, and the use of highly toxic chemicals[3]. In latest years, biological routes for fabrication of nanoparticles have been suggested as possible eco-friendly alternatives to classic chemical and physical methods[58,59]. In particular, green-synthesized silver nanoparticles are emerging as multi-purpose materials, since their biosynthesis is easy and cheap; they are stable over time and effective against different mosquito vectors (*e.g. Ae. aegypti*[60]; recent reviews[25,31]). In addition, Forim *et al.*[21] reported the use of different quantities of neem extracts in nanoparticles formulations, evidencing the enhancement of stability of active compounds and confirming the importance of the use of the appropriate formulation for field utilization.

As regards to neem products and *Aedes* mosquitoes, it has been recently pointed out that polydispersed silver nanoparticles can be rapidly synthesized using neem cake. The nanoparticles were characterized using UV-vis spectrophotometry, Fourier transform infrared spectroscopy, scanning electron microscopy, energy dispersive X-ray spectroscopy, and X-ray powder diffraction analyses. Acute toxicity experiments showed that the neem cake extract and the bio-reduced silver nanoparticles were toxic towards *Ae. aegypti* larvae and pupae. LC_{50} values achieved by the neem cake extract ranged from 106.53 (larva I) to 235.36 ppm (pupa), while LC_{50} achieved by silver nanoparticles ranged from 3.969 (larva I) to 8.308 ppm (pupa)[61]. As regards to neem-synthesized nanoparticles toxic against other mosquito species, silver nanoparticles reduced using the aqueous extracts of leaves and bark of *A. indica* were tested as larvicides, pupicides and adulticides against the malaria vector *An. stephensi* and the filariasis vector *Cx. quinquefasciatus*. The larvae, pupae and adults of filariasis vector *Cx. quinquefasciatus* were more susceptible to silver nanoparticles over *An. stephensi*. The first and the second instar larvae of *Cx. quinquefasciatus* show 100% mortality after 30 min of exposure. The results against the pupa of *Cx. quinquefasciatus* were recorded as LC_{50} 4 ppm after 3 h of exposure. Concerning adult mosquitoes, LC_{50} was 1.06 $\mu L/cm^2$[62].

5. Bio-safety of neem-borne mosquitocides

Neem-based products are usually characterised by moderate toxicity against vertebrates (*i.e.* birds, fishes and mammals)[47,63,64]. The United States Environmental Protection Agency certified the use of cold pressed neem oil as eco-friendly and approved its use with no issues of toxicological, ecological, or environmental concern[65]. However, with regard to neem cake, few analyses of acute and chronic toxicity against non-target aquatic organisms have been conducted[63], even if two exceptions can be considered[31]. First, the acute toxicity evaluation of neem cake extract carried out by Wan *et al.*[66] on juvenile Pacific Northwest salmons, where LC_{50} was 7 mg/L. However, under field conditions, the concentration of neem-based insecticides in a stream unintentionally oversprayed during an aerial- or ground-based operation would unlikely exceed 0.05 mg/L in 15 cm water, even at the highest rate recommended (0.06 kg/ha). On this basis, Wan *et al.*[66] highlighted that the potential of neem cake causing fish kills is small when they are used under product labelled conditions (reviewed in Benelli *et al.*[20]).

Second, the genotoxic effect of silver nanoparticles synthesized using neem cake was studied on *Carassius auratus* (*C. auratus*) using the comet assay and micronucleus frequency test. DNA damage was evaluated on peripheral erythrocytes sampled at different time intervals from the treatment. Interestingly, no significant damages were found at doses below 12 ppm[61]. Furthermore, a single treatment with ultra-low doses of silver nanoparticles synthesized using neem cake did not negatively influence the predation of *C. auratus* fishes against *Ae. aegypti* larvae. Indeed, in standard laboratory conditions, the predation efficiency of a *C. auratus* per day was 7.9 (larva II) and 5.5 individuals (larva III). Post-treatment with sub-lethal doses of silver nanoparticles, the fish predation efficiency was boosted to 9.2 (larva II) and 8.1 individuals (larva III[61].

6. Conclusions and future perspectives

Our review highlighted that neem oil, neem cake, and their fractions, are promising as larvicides, pupicides and oviposition deterrents against important arbovirus vectors belonging to the genus *Aedes*. Notably, the multiple mode of action of neem constituents against insects makes unlikely the development of resistance in mosquitoes[15,16,20]. In addition, neem cake-fabricated metal nanoparticles are easy to produce, stable over time, and may be employed at low dosages to reduce populations of *Ae. aegypti* vectors, with little detrimental effect on non-target mosquito natural enemies[61]. Overall, these findings allowed us to employ neem-borne molecules as an advantageous alternative to build newer and safer *Aedes* control tools, in the framework of Zika virus outbreak prevention[2].

However, despite the encouraging quantity of reported data, several aspects must be clarified to reach the optimum of the success of neem products and their definite consecration. The study of vector-borne diseases is revealing several new aspects, converging into an unexpected system involving several actors with different roles. In the *Xylella* case, a bacterium (*X. fastidiosa*), a vector (*Philaenus spumarius*) and a plant (*Olea europea*) are actively involved[11]. Similarly, recent researches reported the co-occurrence of BTV in different host and vector species, considering that BTV affects sheep much more severely than cattle, and BTV does not eradicate sheep because it cannot persist on midges and cattle alone[67].

Furthermore, most of the production of neem oil and neem cake is obtained in Tamil Nadu, the Indian country of neem, using a simple tool, without any form of purification and rectification. Considering the multipurpose utilization of neem products, their effectiveness against arthropods of public health importance and the variability and complexity of neemome, a lot of work is necessary to perform the adequate product and the method of utilization. An interesting example is the use of neem extracts for red pine weevil management in temperate forestry using a specific device, the "systemic tree injection tube", developed in Canada to inject neem products into trees under pressure[68]. The same system could be utilized also against *X. fastidiosa* invasion.

Conflict of interest statement

We declare that we have no conflict of interest.

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