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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2015.11.013

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

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ARTICLE INFO

Article history:
Received 10 Oct 2015
Received in revised form 28 Oct 2015
Accepted 16 Nov 2015
Available online 20 Apr 2016

Keywords:
Arbovirus
Azadirachta indica
Biosafety
Botanical by-products
Eco-friendly pesticides
Mosquito-borne diseases

ABSTRACT

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

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

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

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

1. Introduction

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

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Foundation Project: Supported by UGC-MRP, New Delhi, India [No. F. No. 36-250/2008 (SR) 24/03/2009] and King Saud University, Saudi Arabia, Vice Deanship of Research Chairs: Research Chairs.

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

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

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

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

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

2. Materials and methods

2.1. Fractionation process of neem cake

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

2.2. HPLC analyses

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

2.3. An. culicifacies rearing

Eggs and larvae of *An. culicifacies* were collected from Department of Rice, All India Co-ordinated Research Project, Rice Research Centre (Tamil Nadu Agricultural University, Coimbatore, India). Following the method reported by Murugan *et al.* [17], the eggs were transferred to laboratory conditions [(27 ± 2) °C, 75%–85% relative humidity, 14:10 (light: dark) photoperiod] and placed in 18, 9, 13, 9, 4 cm plastic containers containing 500 mL of tap water, waiting for larval hatching. Larvae were reared in the plastic containers described above, and fed daily with a mixture of crushed dog biscuits (Pedigree, USA) and hydrolyzed yeast (Sigma–Aldrich, Germany) at 3:1 ratio (w:w). Water was renewed every 2 days. The breeding medium was checked daily and dead individuals were removed. Breeding containers were kept closed with muslin cloth to prevent contamination by foreign mosquitoes. Larvae for experiments were collected daily from