In vitro and *ex vivo* activity of an *Azadirachta indica* seed kernel extract on early sporogonic development of *Plasmodium* in comparison with azadirachtin A, its most abundant constituent

Nisha Dahiya^{1*}, Giuseppina Chianese², Solomon Mequanente Abay^{1,3}, Orazio Taglialatela-Scafati², Fulvio Esposito¹, Giulio Lupidi¹, Massimo Bramucci¹, Luana Quassinti¹, George Christophides⁴, Annette Habluetzel¹, Leonardo Lucantoni^{1,5}

1. *School of Pharmacy, University of Camerino, Piazza dei Costanti, 62032 Camerino, (MC) Italy*

2. *Department of Pharmacy, University of Naples Federico II, Via Montesano 49, 80131 Naples, Italy*

3. *School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia*

4. *Department of Life Sciences, Imperial College London, London, United Kingdom.*

5. *Discovery Biology, Eskitis Institute for Drug Discovery, Griffith University, Nathan, 4111 Queensland, Australia*

* Corresponding author: Nisha Dahiya: nisha.dahiya@unicam.it

Co-authors' email addresses:

ND : nisha.dahiya@unicam.it

GC : g.chianese@unina.it

SMA : solomonabay@gmail.com

OT : scatagli@unina.it

24 FE : fulvio.esposito@unicam.it
25 GL : giulio.lupidi@unicam.it
26 MB : massimo.bramucci@unicam.it
27 LQ : luana.quassinti@unicam.it
28 GKC : g.christophides@imperial.ac.uk
29 AH : annette.habluetzel@unicam.it
30 LL : l.lucantoni@griffith.edu.au

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47 **Abbreviations**

48	ACT: Artemisinin based Combination Therapy
49	AUC: Area Under the Curve
50	AzaA: Azadirachtin A
51	AzaB: Azadirachtin B
52	AzaD: Azadirachtin D
53	AzaI: Azadirachtin I
54	CD1: Cluster of Differentiation 1
55	CDCl ₃ : Deuterated Chloroform
56	CI95: Confidence Interval at the 95% confidence level
57	C _{max} : Maximum serum concentration of a drug
58	<i>P. berghei</i> CTRPp.GFP: <i>Plasmodium berghei</i> strain expressing Green Fluorescent Protein
59	controlled by the Circumsporozoite- and TRAP- related protein promoter
60	DMEM: Dulbecco's Modified Eagle's Medium
61	DMSO: Dimethyl Sulphoxide
62	ESI-MS: Electrospray Ionization Tandem Mass Spectroscopy
63	ESS: Early Sporogonic Stages
64	EtOAc: Ethyl Acetate
65	G6PD: Glucose-6-phosphate dehydrogenase
66	GC-MS: Gas Chromatography- Mass Spectroscopy
67	HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
68	HI-FBS: Heat-inactivated Fetal Bovine Serum
69	HPLC: High Performance Liquid Chromatography
70	i.p.: Intraperitoneal
71	IC ₅₀ : Inhibitory Concentration 50
72	LLINs: Long Lasting Insecticide-treated bed Nets
73	MeOH: Methanol
74	µg/ml: Microgram per Millilitre
75	µM: Micromolar
76	MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
77	NA: NeemAzal®
78	NMR spectroscopy: Nuclear Magnetic Resonance Spectroscopy
79	Ppm: parts per million
80	RBCs: Red Blood Cells
81	RI detector: Refractive Index Detector
82	RPMI: Roswell Park Memorial Institute medium
83	SI: Selectivity Index
84	T _{max} : Time at which maximum plasma concentration of a drug is reached
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Abstract

Background: NeemAzal® (NA) is a quantified extract from seed kernels of neem, *Azadirachta indica* (Meliaceae), with a wide spectrum of biological properties, classically ascribed to its limonoid content. NA contains several azadirachtins (A to L), azadirachtin A (AzaA) being its main constituent. AzaA has been shown to inhibit microgamete formation of the rodent malaria parasite *Plasmodium berghei*, and NA was found to completely inhibit the transmission of *Plasmodium berghei* to *Anopheles stephensi* mosquitoes when administered to gametocytic mice at a corresponding AzaA dose of 50 mg/kg before exposure to mosquitoes.

Purpose: The present study was aimed at i) assessing the pharmacodynamics and duration of action of NA and AzaA against *P. berghei* exflagellation in systemic circulation in mice and ii) elucidating the transmission blocking activity (TBA) of the main NA constituents.

Study design: The NA and AzaA pharmacodynamics on exflagellation were assessed through *ex vivo* exflagellation assays, while TBA of NA constituents was evaluated through *in vitro* ookinete development assay.

Methods: Pharmacodynamics experiments: Peripheral blood from *P. berghei* infected BALB/c mice with circulating mature gametocytes, were treated i.p. with 50 mg/kg and 100 mg/kg pure AzaA and with NeemAzal® (Trifolio-M GmbH) at the corresponding AzaA concentrations. The effect magnitude and duration of action of compounds was estimated by counting exflagellation centers, formed by microgametocytes in process of releasing flagellated gametes, at various time points after treatment in *ex vivo* exflagellation tests. Ookinete Development Assay: The direct effects of NeemAzal® and AzaA on ookinete development were measured by fluorescence microscopy after incubation of gametocytic blood with various concentrations of test substances in microplates for 24 hours.

Results: The exflagellation tests revealed an half-life of NA anti-plasmodial compounds of up to 7 hours at a NA dose corresponding to 100 mg/kg equivalent dose of AzaA. The ookinete development assay showed an increased activity of NA against early sporogonic stages compared to that of AzaA. The IC₅₀ value determined for NA was 6.8 µg/ml (CI95: 5.95 - 7.86), about half of the AzaA IC₅₀ (12.4 µg/ml; CI95: 11.0 - 14.04).

Conclusion: The stronger activity of NA, when compared to AzaA, could not be explained by an additive or synergistic effect by other azadirachtins (B, D and I) present in NA. In fact, the addition of these compounds at 50 µM concentration to AzaA did not evidence any decrease of the IC₅₀ against early sporogonic stages to that obtained with AzaA alone. It is likely that other non-limonoid compounds present in NA may contribute to AzaA activity and enhanced pharmacodynamics against exflagellation both *in vitro* and *in vivo*.

Keywords: NeemAzal[®], Azadirachtin A, pharmacodynamics, exflagellation, *Plasmodium*

1. Introduction

Malaria is a disease causing more than 200 million estimated clinical cases and at least half million deaths annually (WHO, 2015). Currently, malaria control is relying on large scale distribution of long lasting insecticide-treated bed nets (LLINs), aiming at total coverage, and diagnosis-based prompt treatment of malaria cases by artemisinin based combination therapies (ACTs). ACT usage has suffered setback of facing parasite resistance along the Thai-Cambodian border (Noedl *et al.*, 2008) (Wongsrichanalai and Meshnick, 2008), and more recently a delayed response to ACT in some areas of Greater Mekong subregion has been described (WHO, 2014). Ever developing resistance engages researchers to identify new leads for drug development and/or new phytomedicines. At the same time, there is a strong need for the identification of new transmission-blocking agents, able to interrupt malaria transmission. Gametocytes, the sexual stages of *Plasmodium* parasites, are critical for the transmission of the parasite to its vectors. *P. falciparum* gametocytes have been shown to be resistant to common antimalarial drugs and are exceptionally long-lived, thus making clinically cured patients reservoirs of infection (Bousema & Drakeley, 2011). The propagation of the malaria parasites from the vertebrate to the mosquito host occurs through the ingestion of gametocytes by female *Anopheles* mosquitoes during blood feeding. Immediately after reaching the mosquito midgut, mature male and female gametocytes, namely micro- and macrogametocytes, undergo dramatic transformations responding to specific triggers from the midgut environment, including a decrease in temperature (Sinden *et al.*, 1978), an increase in pH (Billker *et al.*, 1997) (Nijhout and Carter, 1978) and presence of xanthurenic acid (Billker *et al.*, 1998), (Nijhout, 1979). Within 10-20 minutes of stimulation, rounded up macrogametes escape the host erythrocytes, and flagellated, motile microgametes form and

disperse (Sinden *et al.*, 1978). Within 24 hours, female gametes fecundated by male gametes develop into round zygotes that mature to elongated motile ookinets and move to the outer midgut surface, completing early sporogonic development. Transmission blocking drug components can be effective in hindering malaria transmission by reducing gametocyte density in the human host (gametocytocidal activity) or by disrupting parasite development in the vector (sporontocidal activity), thus, if widely used, resulting in a reduced number of infective vectors and as a consequence, decreased incidence of malaria cases.

The 8-aminoquinoline primaquine is the only marketed drug reported to clear mature gametocytes within 6 days of exposure in clinical settings (Eziefula *et al.*, 2014). The drug, however, can cause hemolysis in glucose-6-phosphate dehydrogenase (G6PD)-deficient patients, and therefore its extensive usage is not recommended. Artemisinin derivatives target developing gametocytes, most likely by inhibiting heme polymerization and hemoglobin catabolic pathway (Kiszewski, 2010), and have been recently shown to inhibit male gamete formation (Ruecker *et al.*, 2014), however their transmission blocking efficacy in the field has not been confirmed. The identification of novel and safe gametocytocidal/sporontocidal agents is of great public health relevance, since drugs/phytomedicines with such transmission blocking properties may contribute significantly to malaria control. Medicinal plants used for the treatment of malaria in endemic areas have historically been a source for the development of antimalarial medicines; isolated compounds have been used as drug precursors or drug leads. ACTs were developed from a secondary metabolite of *Artemisia annua*, and demonstrate the success of such an approach.

Azadirachtin A, a polyacylated limonoid, is a major secondary metabolite of the seeds of neem,

Azadirachta indica (A. Juss.). This molecule has been found to have neurohormone-like and growth regulatory activities in various insects (Sengottayan, 2013) and to exhibit anti-mitotic effects on cultured insect cells (Salehzadeh *et al.*, 2003). Azadirachtin A and its analogs have been demonstrated to interfere with the exflagellation of malaria parasites (Jones *et al.*, 1994), and to disrupt the assembly of microtubules into axonemes and mitotic spindles during *Plasmodium berghei* microgametogenesis (Billker *et al.*, 2002).

NeemAza[®] (NA), a rich source of azadirachtin A (AzaA), is a quantified alcoholic extract obtained from neem seeds, with a reported limonoid concentration of 57.7%, AzaA being its major constituent (AzaA 34%, azadirachtins B to K 17.7%, salanins 4% and nimbins 2%). We previously demonstrated that NA completely blocks transmission of the rodent malaria parasite *P. berghei* to *Anopheles stephensi* mosquitoes *in vivo*, when administered to gametocytaemic BALB/c mice at a corresponding AzaA dose of 50 mg/kg (Lucantoni *et al.*, 2010). Since AzaA is the major limonoid of this formulation, it is an obvious candidate for being the key molecule conferring the transmission blocking property to the quantified extract. Our previous *in vivo* transmission blocking studies suggested that NA may have stronger transmission blocking activity than AzaA alone, but this hypothesis needed further testing. The study reported here fills this gap by presenting a comparative characterization of the transmission blocking activity of NA and AzaA against the early sporogonic stages (ESS) of *P. berghei*. The study also evaluates for the first time the activity of non-AzaA constituents of NA against the ESS.

Estimation of the pharmacokinetics of bioactive constituents of a plant extract is a challenging task due to the complex chemical interactions of the numerous molecules contained in extracts and the potential generation of multiple metabolites after administration. Given the lack of

knowledge about the metabolites generated after administration of a particular extract, it is difficult to engage HPLC- or GC- based analytical techniques for the determination of common pharmacokinetic parameters (such as C_{max}, T_{max} and AUC) for any particular metabolite. Therefore, it is particularly useful to establish biological response-based assays to understand the pharmacodynamics of whole extracts and their active constituents. In this study, an *ex vivo* assay, which exploits a major target process of AzaA against *P. berghei*, i.e. microgamete formation inhibition of *Plasmodium*, was used to estimate the pharmacodynamics of two varying doses of NA and AzaA.

Materials and Methods

2.1. Animals

Two weeks old, female BALB/c mice weighing 19 ± 3 g were used for the study. Experimental mice were reared and maintained in the animal facility of the University of Camerino (Italy). Experimental animal rearing and handling were in compliance with the Italian Legislative Decree on the “Use and protection of laboratory animals” (D. Lgs. 116 of 10/27/92) and in full adherence with the European Directive 2010/63/UE adopted on 22nd September, 2010.

2.2. Parasite strains

Two strains of the murine malaria parasite *P. berghei*, namely ANKA (chloroquine sensitive) and *CTRPp.GFP* (chloroquine sensitive, pyrimethamine resistant), were used. The *CTRPp.GFP* strain, kindly provided by Prof. R.E. Sinden, is a transgenic line which expresses green fluorescent protein in zygotes, ookinetes and early oocysts (Vlachou *et al.*, 2004). Parasites were maintained in the vertebrate host BALB/c mice and the mosquito host *An. stephensi* through

acyclic and cyclic passages (Lucantoni *et al.*, 2010).

2.3. Cell lines

Human endothelial cell line EA.hy926 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/ml penicillin, 100µg/ml streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (PAA Laboratories GmbH, Austria). Cells were cultured in a humidified atmosphere at 37° C, 5% CO₂.

2.4. Chemicals and reagents

NeemAzal® technical grade (NA, Trifolio-M GmbH, Lahnau, Germany) is a commercial quantified extract from neem (*Azadirachta indica* A. Juss.) seed kernels. According to the producer, the major constituent of NA is AzaA (34%) along with azadirachtins B-K (17.7%) and other non-azadirachtin limonoids (6%). AzaA used in the bioavailability experiments was purchased from Sigma Aldrich (A7430). AzaA and NA fractions employed for the assessment of activity against early sporogonic stages *in vitro* were obtained from NA through chromatography. The NA chromatography conducted in our lab estimated the AzaA content to be 50%, which differed from the producer's specification. Consequently, NA dose (AzaA equivalent) for further experiments i.e. the *in vitro* and *ex vivo* exflagellation experiments and the *in vitro* experiments to estimate the direct effect of NA and AzaA on zygote formation and ookinete maturation, were calculated considering AzaA content as 50%. The corrected NA dosage for *ex vivo* pharmacodynamics experiments was recalculated and indicated in the analysis and graphs.

2.5. Isolation and identification of NeemAzal[®] constituents

NA (1.091g) was subjected to column chromatography over silica gel (70-230 mesh) using a gradient of eluents of increasing polarity from n-hexane/ethyl acetate (EtOAc) 8:2 to EtOAc and then to EtOAc/methanol (MeOH) 1:1 to obtain fourteen different fractions (Table 1). These fractions were analyzed through NMR spectroscopy (Varian Inova 400 MHz) in CDCl₃ (Sigma, USA). The composition of fractions was also analyzed by electrospray ionization tandem mass spectroscopy (ESI-MS) (LTQ OrbitrapXL Thermo Scientific mass spectrometer). Pure fractions were identified by comparing their NMR and MS data with those reported in the literature. Fractions containing mixtures of compounds were further purified through HPLC (Knauer apparatus) on analytical LUNA Si60 (Phenomenex) columns, refractive index (RI) detector. Fatty acids and glycerides present in fractions 1-3 were identified by using GC-MS on a TSQ Quantum GC (Thermo Fischer Scientific) system.

[Insert Fig. 1]

2.6. Pharmacodynamics of NeemAzal[®] and Azadirachtin A using biological response based assay (exflagellation assay)

Eight days prior to the assay, mice (n = 15) were treated intraperitoneally (i.p.) with 120 mg/kg phenylhydrazine to stimulate erythropoiesis (Flanagan and Lessler, 1970). Four days later, the mice were infected i.p. with 10⁷ *P. berghei* (ANKA strain) infected red blood cells (RBCs). On the fourth day after infection, mice were screened for the presence of mature microgametocytes through thin blood smears and microgamete formation was assessed by the exflagellation assay. Five µL of tail blood per mouse were diluted in 120, 140 or 160 µL (based on the haematocrit of

each mouse) of exflagellation medium [RPMI (Sigma, USA), supplemented with 25 mM HEPES and L-glutamate (0.3 g/ml), 25 mM NaHCO₃ and 100 µM xanthurenic acid, pH 8.0]. Eight µL of diluted blood were transferred to a self-made slide chamber consisting of a microscope slide mounted with two coverslips spaced 1 cm apart. Another coverslip was placed on top of the blood drop, covering the edges of the two coverslips to form a chamber. The chamber was sealed with a 1:2 mixture of Vaseline and Tween 80. The slide was incubated for 20 min at 19±1°C and then read under a light microscope (ZEISS Axio Observer.Z1) at 400× magnification, equipped with a 10x10 micrometer grid on the eyepiece. Exflagellation centers – visible as vibrating agglomerations of RBCs around motile microgametes detaching from the microgametocyte residual cell (see figure 4b for an example) - were counted against RBC numbers in grid fields across the diameter of the spread blood drop, reaching 38±2 grid field counts (approximately 30,000 RBCs).

Microgametocyte exflagellation was quantified as:

$$\text{Number of exflagellation centers per 1000 RBCs} = \frac{E}{R * F} * 1000$$

where, E = Total number of exflagellation centers; R = Mean number of RBCs in each grid field

F = Total number of grid fields counted

For the *ex vivo* exflagellation assay, mice demonstrating 7-10 exflagellation centers per 1000 RBCs (preliminarily found to be adequate to obtain sufficient baseline number of exflagellation centers) were selected for the experiment. The exflagellation assay was carried out using pure AzaA and NA, treating experimental gametocytemic mice at AzaA concentrations of 50 or 100

mg/kg body weight. Stock solutions of NA were prepared to a corresponding AzaA concentration of 50 mg/ml with ethanol. Desired test dosages of NA and AzaA were obtained by diluting the stock solution in PBS (pH 6.5), Dimethyl sulphoxide (DMSO), Tween80 and ethanol to obtain a final concentration of 10% DMSO, 7.5% Tween 80, and not more than 20% ethanol. Each mouse received 200 µL of NA or AzaA solution. Doses of NA were prepared referring to the supplier's indication of an AzaA content of 34% in NA.

Chromatographic fractionation of NA conducted at a later stage of the study revealed that the AzaA content of NA was more than 34%, according to our estimates it was about 50%. Consequently, the AzaA equivalent dosage of NA used and reported in the results as 50 mg/kg and 100mg/kg according to our analysis is likely to be about 75 mg/kg and 150 mg/kg respectively. In all the subsequent bioavailability estimation experiments, NA concentrations were prepared based on a 50% AzaA content estimate.

The time points included for the estimation of the number of exflagellation centers were: 0, 15, 30, 45, 60, 90, 120, 180, 240, 300 min after treatment with extract (NA) or molecule (AzaA). At every time-point, a blood drop slide from each experimental mouse was prepared as described, incubated for 20 min and the exflagellation centers counted. Reduction in exflagellation centers due to treatment with the standard extract/molecule was calculated at each time-point using the formula:

Percent inhibition in the formation of exflagellation centers

$$= 1 - \left(\frac{\text{Exflagellation count/1000RBCs at 'nth' time point}}{\text{Exflagellation count/1000RBCs at zero time point}} \right) * 100$$

The exflagellation count at the zero time-point refers to the count obtained immediately before treatment of the mouse with the extract/compound. A maximum of two mice were studied on the

same experimental day. Reduction in the number of exflagellation centers over time was adopted to measure the duration of action of NA and AzaA. The time point showing maximum inhibition (Tmax) corresponded to the time of maximum available concentration of effective metabolites (Cmax).

An *in vitro* exflagellation assay was conducted to estimate the exflagellation inhibitory activity of NA and AzaA at various concentrations, i.e. 50, 25, 12.5, 6.25 and 3.125 µg/ml. Blood from a gametocyaemic mouse was diluted in exflagellation medium containing the test concentration of NA or AzaA. The blood was incubated for 20 minutes and exflagellation centers were enumerated as described above. Exflagellation medium containing DMSO at 0.2% was used as control. Enumeration of exflagellation centers at each concentration and control was recorded in triplicates (3 slides counted for each concentration).

2.7. Ookinete Development assay (ODA)

Experimental BALB/c mice were phenylhydrazine-treated and infected with *P. berghei* CTRPp.GFP as described above. On day four post infection, smears were prepared from the infected mice to screen for number of exflagellation centers as described above. Mice exhibiting 7-10 exflagellation centers per 1000 RBCs were selected for the ODA assay.

Stock solutions of NA and AzaA were prepared at an AzaA concentration of 27.5 mg/ml in DMSO, and were diluted in ookinete medium, i.e. exflagellation medium supplemented with 20% heat-inactivated fetal bovine serum (Sigma, F9665) and 1000 units/ml of penicillin, 1000µg/ml streptomycin (Sigma, P0781), to obtain 50 µg/ml working concentrations. Test solutions were plated onto 96-wells microplates (NUNC), in 6 replicated wells each. DMSO was used as solvent control. Gametocyaemic blood was collected from the selected mice through

cardiac puncture using heparinized syringes and promptly added to the plates, to a final volume of 220 µL/well (1:11 dilution). Microplates were then incubated for 40 hours at 19 °C, to allow for ookinete development to be completed. After incubation, the microplate contents were thoroughly mixed to disaggregate ookinete clumps and diluted 25 times with exflagellation medium into new microplates. Early sporogonic stages (ESS, consisting of zygotes and ookinetes) were counted across the horizontal and vertical diameters of each well under the fluorescent microscope (ZEISS Axio Observer.Z1) (Fig.2). The number of diameters counted varied from 20 to 40 from one well to another depending on ookinete abundance in control wells; counts were carried out on a number of diameters to reach >100 ookinetes per control well. ESS inhibitory activity by each test substance was calculated using the formula:

Percent inhibition in the formation of exflagellation centers

$$= 1 - \left(\frac{\text{Exflagellation count/1000RBCs at 'nth' time point}}{\text{Exflagellation count/1000RBCs at zero time point}} \right) * 100$$

Initial testing of NA was done at 50 µg/ml. Similarly, AzaA and fractions were screened at 50 µg/ml concentrations for activity comparison with NA, whereas AzaB, D and I were tested at 50 µM concentrations (which correspond to 33, 33.8 and 30.9 µg/ml, respectively). Extract or molecules demonstrating >80% inhibitory activity against ESS in at least 3 experiment replicates were considered for IC₅₀ determination. The stock solutions were serially diluted to obtain successive dilutions to determine the IC₅₀. NA and AzaA were tested at 3.125, 6.25, 12.5 and 25 µg/ml. The final DMSO concentration in test and control wells was 0.1% to 0.2%. DMSO did not inhibit ESS at the used concentrations.

[Insert Fig. 2]

2.8. Proliferation assay: estimation of cytotoxicity of NA and AzaA

Proliferation assay was performed using EA.hy926 (Human endothelial, non tumorigenic) cell line to estimate the cytotoxicity of NA and AzaA. The growth inhibitory effect against the cell line was evaluated through MTT assay (Quassinti *et al.*, 2013). Briefly, the cells were seeded in 96-well plates in 100 µl growth medium (2×10^4 cells/ml) and incubated for 24 hours. Then, the cells were exposed to nine two-fold serial dilutions of NA and AzaA, the highest concentration being 200 µg/ml, and incubated for 72 hours. DMSO (0.1% maximum concentration) was used as control.

After 72 hours of incubation, 10 µl MTT (5 mg/ml in phosphate buffered saline) per well were added and the plate was incubated for 4 hours at 37°C. Cell viability was quantified by the ability of living cells to reduce the yellow dye thiazolyl blue tetrazolium bromide (MTT) to a purple formazan product. The formazan product was then dissolved in 100 µl DMSO, after aspirating the medium. Absorbance was measured at 540 nm using a FLUOstar Omega microplate reader (BMG Labtech, Germany). Cell viability was calculated as the percentage of sample absorbance compared to control. IC₅₀ represents the concentration of the compound reducing by 50% cell viability. Selectivity Index (SI) of NA and AzaA was estimated by the formula:

$$SI = \text{mammalian cells IC}_{50} / \text{Plasmodium ODA IC}_{50}$$

2.9. Statistical Analysis

The data were analyzed using Excel (Microsoft Corporation, 2007) and GraphPad Prism 5 (Graphpad Prism Inc.). Student *t* test was used to compare the significance of the difference

between the activity of NA and AzaA or between the fractions or molecules and solvent control. The IC₅₀ values were determined by the four parametric logistic nonlinear regression analysis using GraphPadPrism software, version 5 (GraphPad Software, S. Diego, C.A., USA).

3. Results

3.1. Pharmacodynamics of NeemAzal® and Azadirachtin A

Intraperitoneal treatment of mice with NA at an AzaA concentration of 50 mg/kg resulted in a reduction of about 55% in the number of exflagellation centers 30 minutes post treatment (Fig. 3). The peak value of inhibitory activity (67%; CI₉₅:50 - 84) was observed 45 minutes after treatment. The impact then gradually declined to 20% residual inhibitory activity 3.5 hours after NA treatment.

Treatment of mice with NA at an AzaA equivalent concentration of 100 mg/kg reduced the exflagellation counts by about 70% as soon as 15 minutes after treatment (Fig. 3). This dose produced nearly complete inhibition of exflagellation from 30 minutes up to 180 minutes post treatment. A reduction of more than 50 % was still observed 7 hours after NA treatment at this dose. The measurement of *ex vivo* inhibition of microgamete formation over time can be adopted to relate the effect of NA on *P. berghei* exflagellation in the mouse with the presence of an effective and available concentration of the active constituents in systemic circulation. The response curve of 50 mg/kg revealed that the active components of NA were present in the plasma at a bioactive concentration as early as 30 minutes post-treatment (Fig. 3). The effective concentration of NA reached a maximum (C_{max}) 45 minutes after treatment. Subsequently, the effective dose of the active component(s) dissipated abruptly. A quantifiable inhibitory effect on exflagellation was measurable only for about two hours post-treatment. On the other hand, the

response curve from mice treated with 100 mg/kg of NA showed the presence of an effective concentration of active constituents in the plasma within 15 minutes of treatment. Evidently, at this dose, the active components of NA were present at a concentration capable to produce complete suppression of exflagellation, and their maximal effective plasma concentration was sustained for at least 4 hours post NA administration.

[Insert Fig. 3]

AzaA at 100 mg/kg dosage displayed a much lower inhibitory activity on the formation of exflagellation centers than NA (Fig. 4). A maximum inhibitory value of about 64% (CI₉₅: 50-78), corresponding to AzaA (or its metabolized form) C_{max}, was recorded 60 minutes post-treatment, but immediately after the activity rapidly decreased. At a dosage of 50 mg/kg, no appreciable impact on exflagellation was observed at any time point (Fig. 4).

[Insert Fig.4]

Microscope observation of blood slide preparations from NA and AzaA treated mice revealed not only an impact on the number of exflagellation centers but also on their size and shape (Fig. 5). Samples prepared at time point zero (control) revealed conspicuous, compact, rosette-like exflagellation centers, formed by 15 or more red blood cells sticking to the exflagellating microgametocytes (Fig.5a). Preparations at consecutive time points post treatment (30, 45 and 60 minutes) showed that the arrangement of the centers was altered to poorly formed and dispersed exflagellation centers, constituted usually by less than 10 red blood cells attached to the microgametocyte residual cells (Fig. 5b).

[Insert Fig. 5]

3.2. In vitro inhibition of exflagellation by *NeemAzal*[®] and *Azadirachtin A*

Both NA and AzaA, tested at concentrations ranging from 6.25 to 50 µg AzaA/ml in the *in vitro* exflagellation assay showed a dose dependent inhibitory effect (Fig. 6). At 12.5 µg/ml, NA exerted a significantly higher inhibition (90%) than AzaA (76%) ($p=0.007$). NA still suppressed exflagellation by about 67% ($p=0.05$) at 6.25 µg/ml, whereas AzaA exhibited inconsistent inhibition ranging from 0.7% to 53.2% at this dosage, indicating that the minimum concentration required to achieve a consistent inhibition for NA was 6.25 µg/ml and 12.5 µg/ml for AzaA.

[Insert Fig. 6]

3.3. Characterization of *NeemAzal*[®] fractions and isolation of pure compounds

Silica gel column chromatography of NA yielded about 40 fractions that, on the basis of their TLC behavior, were pooled together to obtain 14 fractions (Table 1).

Fractions 1-3 (13.1 mg, 1.1%) were composed of fatty acids and glycerides; fractions 4-5 (19.6, 1.8 % of total extract) were a mixture of steroids, which were not further investigated.

NMR and MS analysis revealed that fraction 6 was a mixture of azadirachtins A and B and its HPLC purification (Si60 column, hexane-EtOAc 1:1 eluent, flow 0,7 mL/min) afforded these two limonoids in pure state in the following amounts: azadirachtin A: 53 mg, 4.8% of total extract; azadirachtin B: 33 mg, 3.0% of total extract. Fractions 7-9 (eluted with solvents from hexane:EtOAc 4.5:5.5 to hexane:EtOAc 3.5:6.5) were combined and purified by HPLC (Si60 column, hexane-EtOAc 45:55 eluent, flow 0,7 mL/min) affording azadirachtin A (495 mg, 45.2 % of the total extract), azadirachtin B (54.0 mg, 5.0 % of the total extract), Azadirachtin D (27.5 mg, 2.5% of the total extract) and Aza I (22.0 mg, 2.0 % of the total extract) in the pure form. The chemical structures of these compounds are reported in Figure 1.

NMR analysis indicated that the following column chromatography fractions 10-14 (235 mg, 21.5 % of the total extract) did not contain limonids. Given also their inactivity, these fractions

were not further purified and characterized.

3.4 Activity of *NeemAzal*[®] and its constituents on ESS development

Ten of the fourteen fractions obtained from column chromatography were analyzed for activity on ESS development. MS and NMR indicated that Fractions 1-3 contained only fatty acids and/or glycerides. These fractions were analyzed by GC/MS and they proved to contain oleic, palmitic, and linoleic acids and their di- and triglycerides. These primary metabolites, ubiquitous constituents of living cells, including human cells, are unlikely to exert a direct effect on ESS development and therefore we decided to exclude fractions 1-3 from ESS tests. Fractions 6 to 9 exhibited consistent suppression of ESS above 85% at 50 µg/ml (Table 2). In particular, fraction 6, containing mainly AzaA and AzaB, exhibited 88% reduction of ESS. Fractions 7 and 8, which contained almost pure AzaA (>95%), revealed 95% and 93% suppression, respectively. Fraction 9, containing AzaA along with minor amounts of AzaD and AzaI, suppressed ESS development by 94%. Fraction 10-13 did not display appreciable activity.

Fraction 7, which contained 95% AzaA (Fr7) and NA exhibited comparable suppression of ESS at the highest test concentration. When the doses (AzaA equivalents) were reduced, Fr7 evidenced significantly weaker activity than NA on ESS.

[Insert Fig. 7]

At an AzaA concentration of 50 µg/ml, both NA and Fr7 inhibited ESS development by > 90%. At 25 µg/ml, NA and Fr7 reduced ESS development by about 91% and 89%, respectively (Fig. 7). The impact on ESS development was still pronounced in wells containing 12.5 µg/ml of NA (89% inhibition), but in those treated with Fr7 a lower inhibition (35%) was observed ($p =$

0.047). The difference in response between the two products was even more evident at 6.25 µg/ml: at this dosage, NA caused a 40% reduction of ESS, whereas Fr7 at the same concentration revealed no inhibition (3% reduction; $p = 0.001$).

[Insert Fig. 8]

The IC_{50} of NA against ESS was estimated to be 6.8 µg/ml (CI_{95} : 5.95- 7.86) whereas for Fr7 it was 12.4µg/ml (CI_{95} : 11.0- 14.04) (Fig. 8). The activity of NA and AzaA against human endothelial cells (EA.hy 926) showed an IC_{50} value of 45.29 µg/ml (CI_{95} : 40.8- 50.2) and 56.9 µg/ml (CI_{95} : 33.8- 64.3) for NA and AzaA, respectively. This indicates a selectivity index of 9.0 for NA and of 4.6 for AzaA.

To investigate whether the higher activity against ESS of NA compared to Aza A was attributable to the presence of other components in the quantified extract, pure azadirachtins A, B, D, and I, obtained from HPLC purifications, were then tested in ODA, alone and in combination with Aza A.

At a concentration of 50 µM, AzaA exhibited a mean reduction of 83.6 % in ESS development, whereas AzaB, AzaD and AzaI, demonstrated a substantially lower inhibitory activity against ESS development. The average inhibition values obtained for these limonoids were 17%, 7.4% and 21%, respectively.

To investigate a possible potentiating effect of the other azadirachtins on the activity of AzaA, azadirachtin B, D and I were tested at 50 µM in combination with AzaA at IC_{50} concentration i.e. 12.55 µg/ml (17.36 µM). Inhibition values obtained with the combinations did not indicate any synergistic or additive effects (Fig. 9).

[Insert Fig. 9]

4. Discussion

In the present study, the pharmacodynamics of a quantified extract obtained from *A. indica* (NeemAzal[®], NA) on *P. berghei* exflagellation in mice and the inhibitory activity against *P. berghei* early sporogonic stages were compared with those of its main component, Azadirachtin A (AzaA). The duration of action of NA in inhibiting exflagellation centers appeared to be greater than that of AzaA. Furthermore, NA exhibited a higher *in vitro* inhibitory activity against ESS compared to AzaA. Other components of NA, i.e. azadirachtins B, D and I were shown to be much less active than AzaA and they did not appear to confer any synergistic or potentiating effect to AzaA against ESS. This observation is striking in view of the close structural similarity among these limonoids. Although a detailed structure-activity relationship study has not yet been carried out, a comparison of the structures reported in Figure 1 evidences that these compounds differ mainly at the pattern of substitution around the “western” six-membered ring. The integrity of this moiety seems therefore to play an important role in the biological activity of the molecule.

To acquire information on the pharmacodynamics of NA and AzaA, we compared their biological response in *ex vivo* and *in vitro* exflagellation assays. The *ex vivo* results show that, at the same AzaA concentration, NA has an enhanced effect as well as prolonged duration of action compared to AzaA. These observations are in agreement with, the *in vitro* dose-response results showing that NA has a greater potency than AzaA. The overall enhanced inhibitory activity on exflagellation by NA might be explained by the contribution of components other than AzaA present in the quantified extract NA.

Possibly, the non-AzaA azadirachtins and/or other constituents of NA might be protecting AzaA from metabolic degradation, thus improving its *in vivo* effect and sustained exflagellation

inhibitory action over time. NA and AzaA were administered to the mice through the i.p. route, i.e. the same used in our previous work (Lucantoni *et al.* 2010). In mice, the absorption route of a drug administered i.p. is similar to that of orally administered drugs, with the injected constituents being absorbed into the mesenteric vessels which drain into the portal vein and pass through the liver (Turner *et al.*, 2011, Lukas *et al.*, 1971). Therefore, i.p.-injected NA would be subjected to hepatic metabolism before entering the systemic circulation. During this passage through the liver, the structurally similar Aza B, D, I and other limonoids of NA could be protecting AzaA from degradation, for example by competing with AzaA for binding to the cytochrome p450 enzymes. Gedunin, another limonoid isolated from neem fruit, is metabolized by cytochrome p450 enzyme. A gedunin dose of 50 mg/kg for four days caused a 44% *P. berghei* parasitaemia reduction in CD-1 mice, but when administered with 25 mg/kg cytochrome p450 inhibitor dillapiol, an improved parasitemia reduction of 75% was observed (Omar *et al.*, 2003). The *ex vivo* assay described here proved to be a valid approach to understand the time-dependent *P. berghei* exflagellation inhibiting dynamics of Neem Azal and its active constituents.

The interpretation of the *in vivo* efficacy experiments, however, was hampered by the fact that the supplier's estimate of AzaA content in NA was 34%, whereas according to our analysis conducted at a later stage of the study, the AzaA content was in fact about 50%. Initial *ex vivo* exflagellation assays were carried out based on the expected AzaA concentration of 34% in NA. As a result of chromatographic purification of NA, however, it was realized that in the NA batch used for this study AzaA was present at a higher concentration, while the relative content of other azadirachtins (B, D, I) appeared to be lower than declared. Nevertheless, even after

adjusting for the higher concentration of AzaA in NA, the extract still showed a relatively higher inhibitory activity against microgametogenesis and extended duration of action in mice in comparison with the purified limonoid.

A relatively increased activity of NA compared to AzaA was also observed in the *in vitro* ookinete development assay (ODA), measuring impact on early sporogonic stages spanning from gamete formation to ookinete maturation. NA induced about 40% inhibition at an AzaA concentration of 6.25 µg/ml, whereas AzaA achieved a similar inhibition at 12.5 µg/ml. The differences in the *in vitro* exflagellation and ESS inhibitory activity cannot be explained by the hypothesis of protection from metabolic degradation. Rather, a positive interaction between NA constituents, which result in improved activity against the different parasite stages (Lucantoni *et al.*, 2010), might be hypothesized.

Higher activity of whole extracts compared to isolated compounds has been repeatedly reported (Deharo and Ginsburg, 2011). In recent experiments conducted with EtOAc fractions of neem fruit extracts we found that gedunin and azadirone, the main responsible constituents for activity against *P. falciparum* (Chianese *et al.*, 2010) *in vitro* but present in very low amounts, must interact synergistically with each other or with other components of the same phase. *Artemisia annua* extracts have also been shown to possess 6-18 times lower IC₅₀ values against *P. falciparum in vitro* than artemisinin, at a comparable artemisinin concentration (Wright *et al.*, 2010).

To acquire information about possible interactions among the NA constituents, the product was chromatographed and the obtained constituents were screened *in vitro* in the ODA. As expected,

the fractionation yielded AzaA as the major constituent of NA, with azadirachtins B, D and I obtained in smaller amounts. The lack of positive interactions between AzaA and other azadirachtins against ESS, leaves open the possibility that non-azadirachtin constituents of NA might act synergistically with AzaA to confer NA its increased *in vitro* activity on *P. berghei* ESS.

Azadirachtin is known to act on microtubules, including disruption of the organization of mitotic spindles and cytoplasmic microtubule distribution during *Plasmodium* microgametogenesis (Jones *et al.*, 1994; Billker *et al.*, 2002), as well as to cause severe depletion of actin levels in the wing-discs of *Drosophila* (Aritakula *et al.*, 2007). On exposure to AzaA or NA, exflagellation centers are reduced in size due to decreased adherence of surrounding erythrocytes into “rosettes”. Microscopic observation of these rosettes in AzaA- or NA-treated gametocytic blood indicated that the treatment may be interfering with the attachment of RBCs to the microgametocyte residual body during the extrusion process of flagellated microgametes. These observations suggest that, in addition to the known impact on microtubule assembly and organization, NA and AzaA might interfere with the expression and/or function of adhesive proteins during microgametogenesis. The current study supports our previous research works (Lucantoni *et al.*, 2010) in suggesting that NA is more effective than AzaA. Additionally, we recently showed that NA completely inhibits the growth of *P. falciparum* field isolates in *An. coluzzii* mosquitoes at a dose of 70 ppm in direct membrane feeding assays (Yerbanga *et al.*, 2014).

AzaA and NA have an appreciable selectivity index, with oral LD₅₀ > 5000 mg/kg obtained in

587 acute toxicity studies in rats (Niemann and Hilbig, 2000) (Raizada *et al.*, 2001). Additionally,
588 NA showed LD₅₀ > 3365 mg/kg acute toxicity in mice (Niemann and Hilbig, 2000), and
589 azadirachtin technical was shown to have an LD₅₀ > 1500 mg/kg in subchronic oral toxicity
590 experiments in albino rats over 90 days of administration (Raizada *et al.*, 2001). These data
591 strongly suggest that the therapeutic index [TI, the ratio of the highest exposure to the drug
592 which results in no toxicity to the exposure that produces the desired effect (Muller and Milton,
593 2012)] of NA as a transmission blocking phytomedicine would probably be even higher than its
594 selectivity index of 9.0 which we determined *in vitro* using human endothelial EA.hy 926 cells.
595 For a single transmission blocking dose of 100 mg/kg NA, a dose able to suppress
596 microgametogenesis for about seven hours in mice, we would obtain a preliminary TI ≥ 30.
597 Based on the slower specific metabolic rate (the metabolic rate per unit mass) of humans than
598 mice, the actual transmission-blocking duration could be even longer in humans (Sharma and
599 McNeil, 2009). After careful dose scaling and safety studies in humans, a potential transmission
600 blocking phytomedicine based on such a dosage could be developed for resource-limited
601 endemic settings to reduce malaria transmission. There is a current upsurge in utilizing plant
602 extract based libraries for the discovery of new lead molecules for drug development. However,
603 druggability of molecules of plant origin is intricate due to their structural complexity, molecular
604 flexibility, number of stereogenic centers and other limitations which present restraints to the
605 development of ‘drug-like’ analogs (Katiyar *et al.*, 2012), including unfeasible total syntheses
606 (Veitch *et al.*, 2007), thus indicating that the natural source is in many cases the only viable
607 option (Nature News, 2007). Therefore, NA should be viewed as an economic as well as
608 effective choice for the development of a standardized transmission blocking phytomedicine. In
609 this regard, our study also suggests that standardization of the AzaA content, and not of the total

azadirachtins or the total limonoid content, would be opportune.

5. Acknowledgements

This work was supported by EU-FP7 project 223736 TransMalariaBloc, MIUR (PRIN2008: Leads ad Attività Antimalarica di Origine Naturale: Isolamento, Ottimizzazione e Valutazione Biologica) and the University of Camerino, School of Advanced Studies, PhD Programme on Malaria and Human Development . The authors also acknowledge Trifolio-M GmbH, Lahnau, Germany for providing NeemAzal®.

6. Authors' contributions

ND participated in the study design, carried out the experiments, performed the statistical analysis, and drafted the manuscript; GC, SMA, participated in the study design and the execution of the experiments; LQ and MB conducted the cytotoxicity tests. GC and OT conducted the phytochemical analysis of Neem Azal®, GL, GKC, OT and FE contributed to the study design and revision of the manuscript; LL and AH participated in the study design and critically revised the manuscript. All the authors read and approved the final manuscript.

7. Conflict of Interest

The authors have declared no conflict of interest with regard to this work.

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Figure legends

Figure 1: The chemical structures of Azadirachtins A, B, D, I. The structural differences are highlighted in red.

Figure 2: Green fluorescent early sporogonic stages of *Plasmodium berghei* ANKA *CTRPP.GFP* strain; on the left hand elongated ookinetes and on the right hand spherical zygotes. In the ookinete development assay > 95% of zygotes complete ookinete formation within 24 hours and strongly express green fluorescent protein at least up to 40 hours of incubation.

Figure 3: Activity over time curves obtained by plotting percent reduction in number of exflagellation centers with respect to untreated controls as a function of time elapsed after treatment. The NA 50 mg/kg and NA 100 mg/kg values cumulate data from 15 and 6 mice,

respectively. Vertical bars represent 95% confidence intervals. NA= NeemAza[®]. Data points without confidence intervals represent reading recorded from only one mouse for that particular time point.

Figure 4: Activity over timecurves of AzaA at 50 mg/kg and 100 mg/kg. The curves cumulate data obtained from 6 mice each. Vertical bars represent 95% confidence intervals. AzaA= Azadirachtin A. Data points without confidence intervals represent readings recorded from only one mouse for that particular time point.

Figure 5: NA effects on exflagellation center morphology.

a) Exflagellation centers at zero time point of NA treatment (control): the centers are of varying size but easily recognizable as vibrating aggregations of 15 or more RBCs around exflagellating microgametocytes.

b) Exflagellation centers 30 minutes after NA (100 mg/kg) treatment: Exflagellation centers appear smaller, frequently formed by less than 10 RBSs aggregating loosely around microgametes extruding from microgametocyte residual cells. The circles and arrows indicate affected exflagellation centers, which are small and dispersed.

Figure 6: Reduction in the number of exflagellation centers by NA and AzaA at various concentrations *in vitro*. The products were added to gametocyaemic mouse blood, and exflagellation centres counted after 20 minutes of incubation. Mean values of triplicate samples. Vertical error bars represent 95% confidence intervals, and p values for each concentration are indicated.

Figure 7: Impact of different dosages of NA and Fr7 (95% AzaA) on ESS (Early Sporogonic Stages: zygotes and ookinetes) in the Ookinete Development Assay (ODA). Mean % inhibitions from four independent replicate experiments, each consisting of 6 replicate wells. Vertical error bars represent 95% confidence intervals. NA=NeemAza[®]), Fr7 AzaA= fraction 7 containing 95% Azadirachtin A, was obtained from column chromatography of NA.

Figure 8: IC₅₀ (inhibitory concentration 50) obtained by grouping % Inhibition values from 3 independent replicates, each concentration tested in sextuplicates (wells). NA = NeemAza[®], fr7 AzaA= fraction 7 containing 95% Azadirachtin A, was obtained from column chromatography of NA

Figure 9: ESS inhibition by AzaA, B, D, I. and binary combinations thereof. AzaA was tested at its IC₅₀ value (12.55 µg/ml = 17.36 µM) while Aza B, D and I were tested at 50 µM. In wells receiving combinations, the same concentration of each compound was tested. Mean values of % inhibitions were obtained from four independent replicate experiments (6 replicates each). Vertical error bars represent 95% confidence intervals.

Figures

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(a)

(b)

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