Journal of Ethnopharmacology 175 (2015) 287–294

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Effect of neem (*Azadirachta indica* A. Juss) leaf extract on resistant *Staphylococcus aureus* biofilm formation and *Schistosoma mansoni* worms

Patrick V. Quelemes [a], Márcia L.G. Perfeito [a], Maria A. Guimarães [a], Raimunda C. dos Santos [a], David F. Lima [a,b], Carlos Nascimento [c], Marcos P.N. Silva [d], Maria José dos S. Soares [e], Cristina D. Ropke [f], Peter Eaton [a,g], Josué de Moraes [d], José Roberto S.A. Leite [a,*]

[a] Biodiversity and Biotechnology Research Center, Biotec, Federal University of Piauí, UFPI, 64202020 Parnaíba, PI, Brazil
[b] Federal University of Vale do São Francisco, UNIVASF, Paulo Afonso, BA, Brazil
[c] Laboratory of Parasitology, Instituto Butantan, São Paulo, SP, Brazil
[d] Center for Research on Neglected Diseases, University of Guarulhos, Guarulhos, SP, Brazil
[e] Department of Veterinary Morphophysiology, Federal University of Piauí, Teresina, PI 64049550, Brazil
[f] Phytobios, Pesquisa, Desenvolvimento e Inovação, Barueri, SP, Brazil
[g] UCIBIO, REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, 4169-007 Porto, Portugal

ARTICLE INFO

Article history:
Received 4 March 2015
Received in revised form
5 August 2015
Accepted 19 September 2015
Available online 25 September 2015

Keywords:
Azadirachta indica
Neem leaf extract
Biofilm
Schistosoma mansoni

ABSTRACT

Ethnopharmacological relevance: There are ethnopharmacological reports supporting the use of neem (*Azadirachta indica* A. Juss) leaf against bacterial and worm infections. However there is a lack of studies about its effect on bacterial biofilm formation and *Schistosoma mansoni* worms. This study reports the *in vitro* effects of neem leaf ethanolic extract (Neem EE) on Methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm and planktonic aggregation formation, and against *S. mansoni* worms.

Materials and methods: Quantification of the Azadirachtin (AZA), thought to be one of their main compounds related to biological effects, was performed. The effect of sub-inhibitory concentrations of Neem EE on biofilm formation and planktonic aggregates of *S. aureus* was tested using the crystal violet dye method and atomic force microscopy (AFM) analysis, respectively. Changes in *S. mansoni* motor activity and death of worms were analyzed *in vitro* after exposition to the extract. Treated schistosomes were also examined using confocal laser scanning microscopy.

Results: It was observed the presence of AZA in the extract (0.14 ± 0.02 mg/L). Testing Neem EE sub-inhibitory concentrations, a significant biofilm adherence inhibition from 62.5 μg/mL for a sensitive *S. aureus* and 125 μg/mL for two MRSA strains was observed. AFM images revealed that as the Neem EE concentration increases (from 250 to 1000 μg/mL) decreased ability of a chosen MRSA strain to form large aggregates. In relation of anti-*schistosoma* assay, the extract caused 100% mortality of female worms at a concentration of 50 μg/mL at 72 h of incubation, while 300 μg/mL at 24 h of incubation was required to achieve 100% mortality of male worms. The extract also caused significant motor activity reduction in *S. mansoni.* For instance, at 96 h of incubation with 100 μg/mL, 80% of the worms presented significant motor activity reduction. By the confocal microscopy analysis, the dorsal surface of the tegument of worms exposed to 300 μg/mL (male) and 100 μg/mL (female) of the extract showed severe morphological changes after 24 h of treatment.

Conclusions: Neem leaf ethanolic extract presented inhibitory effect on MRSA biofilm and planktonic aggregation formation, and anthelmintic activity against *S. mansoni* worms.

Abbreviations: AFM, atomic force microscopy; ATCC, American Type Culture Collection; AZA, azadirachtin; CFU, colony forming unit; CLSI, Clinical and Laboratory Standards Institute; DMSO, dimethyl sulfoxide; FAA, formalin-acetic acid-alcohol solution; FT-MS, Fourier transform mass spectrometry; HPLC-FT-MS, liquid chromatography coupled to ultra-high-resolution mass spectrometry; MIC, minimum inhibitory concentration; Neem EE, neem leaf ethanolic extract; NMR, nuclear magnetic resonance; PZQ, praziquantel; SPE, solid-phase extraction; TSB, tryptic soy broth

* Corresponding author.
E-mail address: jrsaleite@gmail.com (J.R.S.A. Leite).

http://dx.doi.org/10.1016/j.jep.2015.09.026
0378-8741/

1. Introduction

Various parts of the neem tree *(Azadirachta indica* A. Juss), a member of Meliaceae family, have been used for centuries as part of traditional medicine in various locations around the world, especially in India and South Asia (Biswas et al., 2002; Subapriya and Nagini, 2005). There are ethnopharmacological reports supporting their use against bacterial and worm infections by the oral use of their leaves decoction, for instance (Agyare et al., 2014; Sharma et al., 2014). Thus, the antimicrobial properties of their extracts and compounds are among the most widely studied pharmacological aspects. Although not definitively described, the antimicrobial action could be due the presence of the tetranortriterpenoid compound azadirachtin, which is present in neem seeds and leaves, among other potential agents that have been characterized such as: mahmoodin, nimbolide, nimbin and salannin (Siddiqui et al., 1992; Dai et al., 1999; Biswas et al., 2002; Sharma et al., 2003).

The effects of neem extracts against bacteria (planktonic forms) (Fabri et al., 1998; Okemo et al., 2001; Alzoreky and Nakahara 2003; Chomnawang et al., 2009), bacterial biofilm (Harjai et al., 2013; Geethashri et al., 2014), dental plaque (Wolinsky et al., 1996; Pai et al., 2004; Botelho et al., 2008), *Candida albicans* fungi (Okemo et al., 2001; Polaquini et al., 2006); viruses (Parida et al., 2002; Faccin-Galhardi et al., 2012), and parasites such as the *Plasmodium sp.* that cause malaria (Badam et al., 1987; Vasanth et al., 1990), have been described to date.

Regarding antibacterial activity, effects of neem extracts on planktonic bacteria have been well reported. However, the possible action of neem on virulence factors such as biofilm and planktonic aggregation formation deserves better attention. The opportunistic human pathogen *Staphylococcus aureus*, for example, which has recognized importance in severe hospital infections, a well-known capacity to form biofilms and the ability to acquire resistance to antibiotics (Nicaido, 2009), is still a challenge for researchers in the search for agents with efficacy against it and in particular against their biofilms (Tang and Stratton, 2010).

In addition, to date no reports concerning the effects of neem extract on the helminth *Schistosoma mansoni* that causes schistosomiasis have been published. This condition has been described as a disease of poverty since it still affects hundreds of millions of people, and is more prevalent among the poor than in wealthy people and constitutes a global public health problem (Steinmann et al., 2006). Nowadays, schistosomiasis control is almost entirely based on chemotherapy using praziquantel, which is effective against all species of *Schistosoma* (Hagan et al., 2004). However, repetitive use of the drug can lead to resistance and tolerance by parasites in the wild in different areas and by different strains in the same area, reaffirming the need to develop new treatment for schistosomiasis (Pica-Mattoccia and Cioli, 2004; Doenhoff et al., 2008).

This study reports the effect of neem leaf ethanolic extract on Methicillin-resistant *S. aureus* (MRSA) biofilm and planktonic aggregation formation and against *S. mansoni* worms.

2. Materials and methods

2.1. Plant material, preparation of extract and chemical analysis

Leaves of neem were collected from the Anidro do Brasil Extrações S.A. Company Farm., Parnaíba city, Piauí state, Brazil (latitude 03°01'27, 5″ S and longitude 41°44'53, 5″ W). The material was collected in the morning of March 2013 and dried in the sun until 6.08% humidity. Specimens were deposited at Herbarium "Delta do Parnaíba" at Federal University of Piauí (Voucher number: HDELTA 1335).

The neem leaf ethanolic extract (Neem EE) was obtained by maceration in 95% PA ethanol for 7 days, with 3 renewals of the extract liquid. The extract was filtered and concentrated using a rotary evaporator at controlled temperature and vacuum of 60 °C and 175 mbar, respectively. The extract represented 12.5% yield from 150 g of dried plant material and was dissolved in a solution of dimethyl sulfoxide (stock solution: 10% v/v in water) for biological assays, in which the highest DMSO concentration used in the *in vitro* tests did not exceed 1% after the dilution in the culture media.

The qualitative and quantitative analysis of azadirachtin (AZA) in the Neem EE was performed by liquid chromatography coupled to ultra-high-resolution mass spectrometry (HPLC-FT-MS, Bruker) and liquid chromatography coupled to off-line nuclear magnetic resonance spectroscopy (HPLC–SPE/NMR, Bruker). The pattern of AZA was selectively isolated by HPLC–SPE and its characterization and purity determined by ultra-high resolution mass spectrometry (FT-MS) and 1H-NMR. After confirming the identity and purity of the isolated AZA, it was used for construction of the calibration curve for the quantification of AZA in the Neem EE.

2.2. Bacterial strains and culture conditions

The effect of Neem EE on four bacterial strains of *S. aureus* was determined: one was Methicillin-sensitive (MSSA)-ATCC 29213; and three strains were Methicillin-resistant (MRSA)-Col, WB69 and USA100. Bacteria were grown in Müller–Hinton agar for 24 h and, from isolated colonies, a microbial suspension was standardized with sterile saline to turbidity equivalent to 0.5 on the McFarland scale (approximately $1–2 \times 10^8$ CFU/mL). This solution was diluted ten times and used in the *in vitro* experiments described below, where each well in the 96-well plate contained the bacteria at a final concentration of 5×10^5 CFU/mL.

2.3. Inhibition of S. aureus biofilm adherence assay

Previously, minimum inhibitory concentration (MIC) was determined according Clinical and Laboratory Standards Institute (2012) and Sufian et al. (2013), where 2-fold dilution series with the Neem EE at concentrations ranging from 250 to 2000 μg/mL in a 96-well plate was performed. MIC was defined as the lowest concentration of Neem EE that restricted bacterial growth of *S. aureus* strains in the culture media. Then the effect of sub-inhibitory concentrations of Neem EE was evaluated following Xiao et al. (2007) and Hochbaum et al. (2011), with adaptations. Strains were grown in 96-well plates with Tryptic Soy Broth (TSB) with 0.5% of glucose and Neem EE at concentrations of 1/2, 1/4, 1/8, 1/16 MIC. After 24 h, the wells were rinsed with saline solution and the cells that remained adhered in the wells were fixed with methanol P.A. for 10 min. After removal of the methanol, wells were stained with 100 μL of 0.1% crystal violet (CV) dye for 5 min, rinsed twice with distilled water, and thoroughly air-dried under ambient conditions. For biofilm adherence quantification, 100 μL of ethanol (95%) was added to the CV-stained wells for 30 min. The resulting solutions were transferred to other 96-well plates, and their absorbance (A) was measured at 595 nm. This assay was performed in quadruplicate. The percentage of inhibition was calculated using the equation: $(1 - A_{595}$ of the test/A_{595} of non-treated control$) \times 100$. Data were expressed as mean ± SD and evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using GraphPad Prism 5.0. $^{*}p < 0.05$ or $^{***}p < 0.001$ was considered statistically significant, compared to the control group consisting of a non-treated control biofilm.

2.4. Atomic force microscopy analysis

Effect of Neem EE on USA100-MRSA planktonic aggregates was examined by Atomic Force Microscopy (AFM). A similar methodology to that previously described for the biofilm adherence assay (item 2.3) was performed, where the microorganism was incubated with the extract at 1/2, 1/4 and 1/8 MIC, in TSB with 0.5% of glucose. The non-treated inoculated culture media was used as a negative control. After incubation for 24 h, 15 μL of the culture media containing the treated or non-treated bacteria were deposited onto a clean glass surface followed by air-drying. The samples were then gently rinsed with 1 mL of deionized water to remove salt crystals and air-dried again under ambient conditions before analysis. All samples were prepared at the same time, accurately exposed to the same conditions and examined within 12 h of deposition. AFM was carried out with a TT-AFM microscope from AFM Workshop (USA). The analysis of Neem EE effect on planktonic bacterial aggregate morphology was carried out in vibrating mode, using cantilevers (Tap300-G/Budget Sensors) with resonant frequency approximately 300 kHz. Multiple areas of each sample were examined, but here we show only representative images. Images were analyzed using Gwiddion software 2.33.

2.5. Anti-Schistosoma assay

Adult *S. mansoni* worms (BH strain) were obtained by perfusion of hamsters (Smithers and Terry, 1965), 7 weeks after infection with cercariae. For the *in vitro* test, after washing, schistosomes were incubated in a 24-well culture plate, placing one coupled worm pair in each well, containing the RPMI 1640 medium at 37 °C in a 5% CO_2 atmosphere as previously described (Moraes et al., 2011; de Moraes et al., 2013a). Neem EE was used to obtain final test concentrations of 50 to 300 μg/mL in the culture plates with final volume of 2 mL. The parasites were incubated for 120 h and monitored every 24 h using an inverted optical microscope and × 2.5 and × 10 objective lenses. Neem EE effect was assessed with emphasis on changes in worm motor activity and death of worms as previously described (de Moraes et al., 2013b). All experiments were carried out in triplicate or quadruplicate and were repeated at least three times. Control worms were assayed in only RPMI 1640 medium as the negative control group and a solvent control with RPMI 1640 medium plus 0.5% DMSO was also performed. The medium supplemented with 1 μg/mL of praziquantel (PZQ) was the positive control group.

The present study was approved by the Ethics Committee at Universidade Estadual de Campinas, SP, Brazil (approval number 2753-1). All the animals were handled in strict accordance with good animal practice as defined by the Faculty of Sciences of Guarulhos for animal husbandry, according to with the Brazilian legislation (Comissão de Ética de Uso de Animais, CEUA, 11,794/2008).

2.6. Confocal laser scanning microscopy analysis

To observe morphological changes in the tegument of adult worms after the *in vitro* assay, schistosomes were examined using a confocal laser scanning microscope following standard procedures presented elsewhere (de Moraes et al., 2012). Briefly, at the end of the drug treatment period (120 h) or in the case of death, the worms were fixed in a formalin–acetic acid–alcohol solution (FAA) and analyzed under a confocal microscope (Laser Scanning Microscope, LSM 510 META, Carl Zeiss, Standorf Göttingen, Vertrieb, Germany). Autofluorescence was excited with a 488 nm line from an Argon laser, and emitted light was collected with 505 nm (de Moraes et al., 2014).

3. Results and discussion

3.1. Chemical analysis

This work describes two effects of neem leaves (Fig. 1A), inhibition of MRSA biofilm and planktonic aggregation and antihelmitic action against the *S. mansoni* worm. We did not aim to describe a complete phytochemical analysis of Neem EE, instead, we seek to quantify one of the main compounds involved in antimicrobial effects. It was evaluated the presence of AZA (azadirachtin), which is thought to be the major bioactive compound in neem extracts (Dai et al., 1999; Sharma et al., 2003). AZA (Fig. 1B) belongs to the class of limonoids, and is present as a secondary metabolite in both neem seeds and leaves, but is most commonly extracted from its seeds (Sharma et al., 2003). It is a highly oxidized tetranortriterpenoid with a complex molecular architecture with a diverse array of oxygenated groups (Veitch et al., 2007) and considerable variability of its content in plants has been reported (Sidhu et al., 2003).

Qualititative analysis showed that the AZA was present in the extract evaluated in this work. The ion chromatogram showed a molecular ion at m/z 719.25 for a standard solution of AZA isolated by SPE/HPLC obtained for the chromatographic band eluted at 6.82 min. The compound has the following signs of hydrogens for the 1H-NMR spectrum: 4.76 (m, H-1); 2.32 (m, H-2a); 2.26 (tl, 3.3 Hz, H-2b); 5.50 (tl,2.8 Hz, H-3); 3.35 (d, 12.5 Hz, H-5); 4,61 (dd, 12.5; 2.7 Hz, H-6); 4.76 (m, H-7); 3.34 (m, H-9); 4.67 (dl, 3.3 Hz, H-15); 1.68 (m, H-16 a); 1.32 (d, 13.1 Hz, H-16b); 2.38 (dl, 5.2 Hz, H-17); 2.01 (sl, H-18); 4.15 (d, 9.7 Hz, H-19a); 3.63 (d, 9.7 Hz,

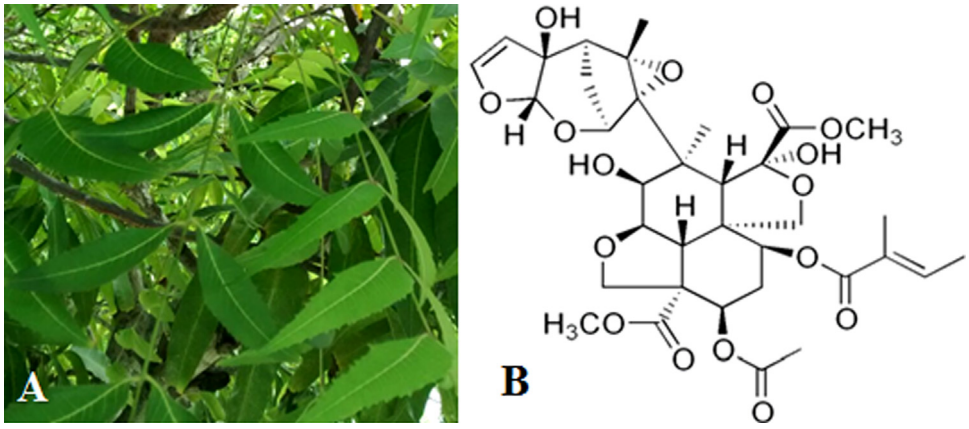

Fig. 1. (A) Leaves of neem (*Azadirachta indica* A. Juss); (B) planar chemical structure of azadirachtin molecule. (Photo: Raimunda C. dos Santos).

H-19b); 5.65 (sl, H-21); 5.05 (d, 2.9 Hz, H-22); 6.46 (d, 2.9 Hz, H-23); 4.08 (d,8.9 Hz, H-28 a); 3.77 (d, 8.9 Hz, H-28b); 1.75 (s, H-30); 2.87 (s, OH-7); 5.04 (s, OH-11); 2.94 (s, OH-20); 3.69 (s, OMe-12); 3.80 (s, OMe-29); 1.95 (s, OAc-3); 6.95 (m, H-3'); 1.78 (dd, 7.1; 0.8 Hz, H-4') and 1.85 (m,H-5') which are correlated with the identity of AZA.

After identifying and performed the calibration curve, the observed content of AZA in Neem EE was determined to be 0.14 ± 0.02 mg/L, which is consistent with secondary metabolites identified in ethanolic extracts of leaves. Here we emphasize that is probable that the biological effects described below may be caused by the synergistic action with several other compounds present in the extract.

3.2. Effect of neem EE on S. aureus

Biofilm formation is an important bacterial virulence factor that can affect prosthesis, tubes and, mainly, cardiac valves causing serious problems in medical practice, requiring very high doses of antibiotics to prevent recurrence (Donlan and Corteston, 2002; Hoiby et al. 2010). This aspect is even more serious in the case of infection by resistant bacteria such as MRSA, leading to a requirement for new agents that act on this microorganism or on its virulence mechanisms (Tang and Stratton, 2010). To evaluate Neem EE effect on biofilm formation, the resistant microorganisms selected for this study are community and/or hospital-acquired bacterial strains recognized by their pathogenicity and virulence (Jevons, 1961; Ribeiro et al., 2005; Roberts, 2013). The antibacterial test using Neem EE showed a MIC of 1000 μg/mL for the MSSA strain. This result is according to other works that evaluates extracts of different parts of the neem tree. Fabri et al. (1998) tested stem bark methanolic extract and observed a MIC of 1000 μg/mL; Alzoreky and Nakahara (2003) tested methanolic extract of flowers that showed MIC of 1320 μg/mL. For all MRSA strains, we observed a MIC of 2000 μg/mL. Chomnawang et al. (2009) observed a MIC of 5000 μg/mL for MSSA and no effect for MRSA. The concentration of DMSO used to dissolve the extract, after diluted in culture media, did not cause any bacterial inhibition in the test, and all tested strains presented the predicted susceptibility to the standard antibiotics recommended by CLSI (Oxacilin, MIC < 0.5 μg/mL for MSSA and Vancomycin, MIC = 1 μg/mL for MRSA strains). Overall, these results are in line with previously reported data and suggest that while Neem EE possesses antibacterial properties against common *S. aureus* strains, the concentrations required for inhibition are many times greater than the effective concentrations of standard antibiotics.

On the other hand, the inhibition adherence assay using Neem EE sub-MIC concentrations showed that the extract was capable of inhibiting biofilm formation on all tested strains. On ATCC 29213-MSSA; WB69-MRSA and USA100-MRSA, this effect was significant using as little as 1/16 the MIC (Fig. 2A, C and D). At 1/2 MIC, the extract showed more than 80% adherence inhibition for the ATCC 29123-MSSA and USA100-MRSA strains (Fig. 2A and D).

Atomic force microscopy was performed to observe the aggregation behavior of MRSA cells with and without Neem EE at sub-MIC concentrations. Fig. 3 shows AFM images illustrating the effect of Neem EE sub-inhibitory concentrations on USA100-MRSA planktonic aggregates. This microorganism was selected for this test since the previous experiment showed it to be more susceptible to the anti-biofilm action of the extract (Fig. 2D).

S. aureus is known to grow in small clusters of 5 to 20 cells but in some circumstances, it can assemble in large aggregates, *via*

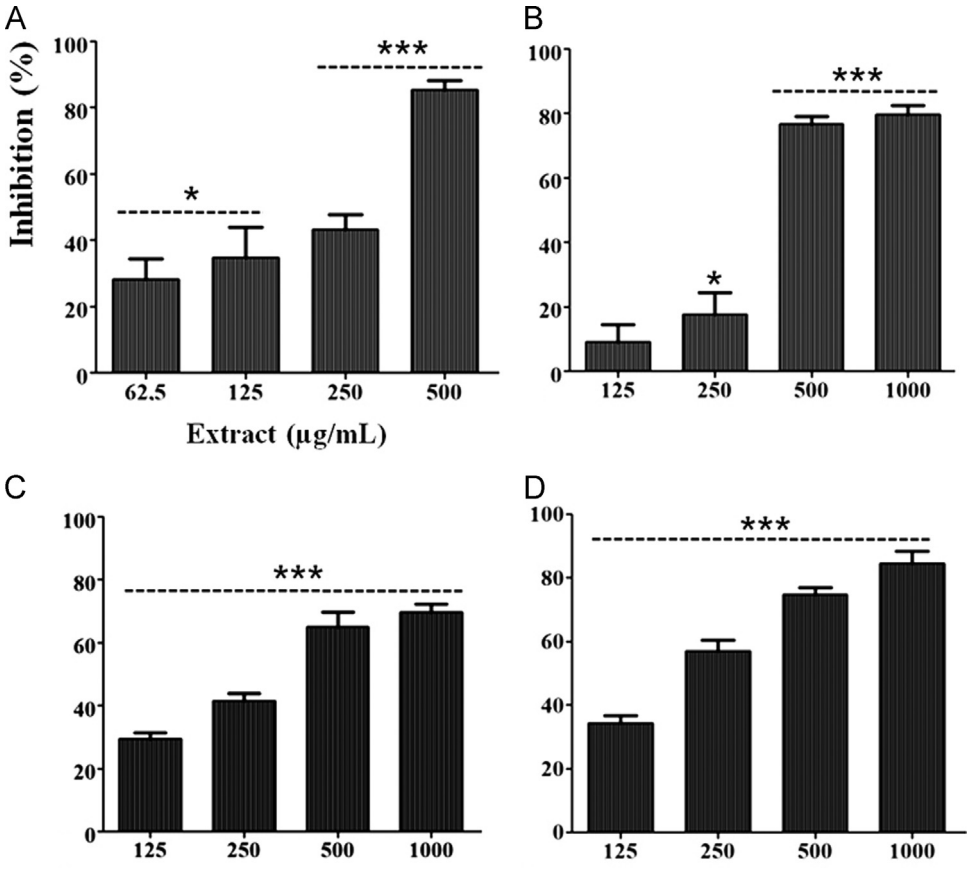

Fig. 2. *In vitro* effect of Neem EE sub-inhibitory concentrations on *S. aureus* biofilm adherence. (A) ATCC 29123-MSSA; (B) Col-MRSA; (C) WB69-MRSA and (D) USA100-MRSA. Each column represents the mean ± SEM; $^{*}p < 0.05$, $^{***}p < 0.001$, compared to the control group.

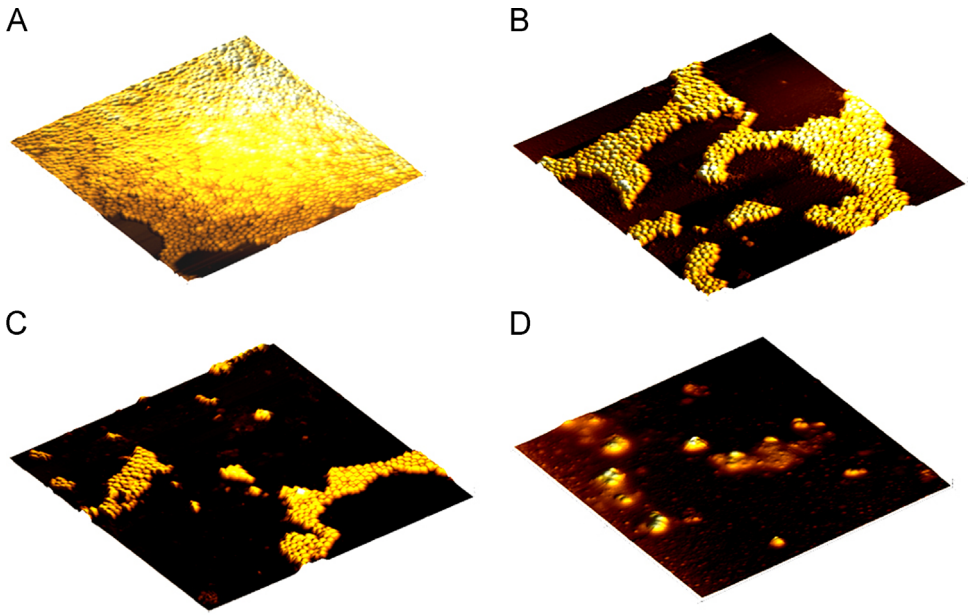

Fig. 3. Representative AFM images of the effect of Neem EE sub-MIC concentrations on USA100-MRSA planktonic aggregation. (A) Control (non-treated); (B) 250 μg/mL; (C) 500 μg/mL and (D). 1000 μg/mL. In all images, the *X* and *Y* axes are 40 μm; the *Z* axis is 2.2 μm.

polysaccharide intercellular adhesin (PIA) formation (Merino et al., 2009; Haaber et al., 2012). Fig. 3A shows a large aggregate of *S. aureus* induced by the presence of glucose in the culture media, which was added as a supplement to encourage the multicelullar aggregation step of biofilm formation (You et al., 2014). According to Haaber et al. (2012), bacterial cells in aggregates are protected against antimicrobial compounds and, in contrast to biofilms, maintain high levels of metabolic activity and mobility. This combination may contribute to the difficulties of eradicating infections. However, as is the case with biofilms, it is known that the aggregation process can be influenced by sub-lethal concentrations of antibiotics (Mirani and Jamil, 2011).

Analyzing the AFM images, we note that as the Neem EE concentration increases, smaller aggregates were observed, reflecting decreased ability of microorganisms to form large aggregates (Fig. 3B–D). In Fig. 3D, it was observed some non-aggregated microorganisms. The loss of the ability to form aggregates in addition to the decreased capacity to promote the biofilm initial adhesion (Fig. 2) strongly suggests that that the Neem EE has the ability to interfere to these important MRSA virulence factors.

Although there are many reports describing neem antibacterial activity, this study evaluated the effect of its leaf extract on MRSA biofilm and planktonic aggregation formation. This capacity may have been possible due to the presence in the extract of compounds such as tetratriterpenoids, including azadirachtin (Jarvis et al., 1999). These compounds may be able to inhibit the *S. aureus* production polysaccharide intercellular adhesin due inhibition of *ica* genes expression (Mack et al., 2004; Atshan et al., 2012) or could have acted on *S. aureus* cell wall hydrophobicity (Kustos et al., 2003). Further studies to test the fractions and molecules that comprise the Neem EE to elucidate its antimicrobial and antibiofilm action mechanism would be required to clarify these aspects.

3.3. Effect of neem EE on S. mansoni

The drug of choice for the treatment and control of schistosomiasis is praziquantel-PZQ (Hagan et al., 2004). However, some evidence of PZQ resistance in the wild, as well as reports of resistance in laboratory experiments have been reported (Fallon and Doenhoff, 1994; Ismail et al., 1996). In this context, the research and development of new and more effective antischistosomal compounds is required. This study is the first one investigating the *in vitro* efficacy of Neem EE against adult worms of *S. mansoni*.

The effect of Neem EE on the viability of the adult worms was analyzed with respect to incubation time and concentration. The results demonstrate that the extract effects are dose-dependent. As shown in Table 1, all adult worms were dead after 24 and 96 h of incubation with Neem EE at a concentration of 300 and 200 μg/mL, respectively. The worms remained viable in the negative and solvent control groups (RPMI or RPMI with 0.5% DMSO), but the positive control (1 μg/mL of PZQ) caused the death of all parasites within 24 h. During monitoring of the cultures, no motor activity changes in adult worms were observed for the negative control groups, while the positive control (PZQ) showed complete loss of motor activity of the worms. The addition of Neem EE to the culture medium resulted in significant motor activity reduction in *S. mansoni.* For instance, at 96 h of incubation with 100 μg/mL of Neem EE, 80% of the worms presented significant motor activity reduction. That paralysis is associated to important neurotransmitters or neuromodulators such as acetylcholine, dopamine, serotonin, among others (Geary et al., 1992; Sangster et al., 2005).

Incubation of adult *S. mansoni* with Neem EE at doses higher than or equal to 50 μg/mL kept the male and female adult worms separated, which prevented the mating process and further oviposition. Furthermore, it was observed that the extract acted preferably against female worms instead of male worms. Fig. 4 shows that the Neem EE caused 100% mortality of female worms at a concentration of 50 μg/mL at 72 h of incubation, while a concentration of 300 μg/mL at 24 h of incubation was required to achieve 100% mortality of male worms. Similar results in relation to the difference of susceptibility between male and female schistosomes, both *in vitro* and *in vivo* were observed with various antischistosomal drugs. For example, Mitsui et al. (2009) reported that female worms of *S. mansoni* were often more susceptible than males to artenusate *in vitro*. de Moraes et al. (2014) reported that female adult worms were more affected by phytol than male adults when the drug was administered orally to mice infected with adult *S. mansoni*. The literature is unclear in relation to difference in the drugs susceptibility between males and females of *S.*

Table 1
In vitro effects of Neem EE on *S. mansoni* adult worm.

Group	Period of incubation (h)	Dead worms (%)[a]	Motor activity reduction (%)[a]		Worms with tegumental alterations (%)[a]	
			Slight	Significant	Partial	Extensive
Control[b]	24	0	0	0	0	0
	48	0	0	0	0	0
	72	0	0	0	0	0
	96	0	0	0	0	0
	120	0	0	0	0	0
0.5% DMSO[c]	24	0	0	0	0	0
	48	0	0	0	0	0
	72	0	0	0	0	0
	96	0	0	0	0	0
	120	0	0	0	0	0
PZQ [d]	24	100	0	100	0	100
	48	100	0	100	0	100
	72	100	0	100	0	100
	96	100	0	100	0	100
	120	100	0	100	0	100
Extract 50 μg/mL	24	0	0	0	0	0
	48	0	0	0	0	0
	72	50	0	50	0	50
	96	50	0	50	0	50
	120	50	0	50	0	50
Extract 100 μg/mL	24	50	0	50	0	50
	48	50	0	50	0	50
	72	50	0	50	0	50
	96	50	0	80	20	80
	120	50	0	100	0	100
Extract 200 μg/mL	24	50	0	50	0	50
	48	50	0	50	0	50
	72	50	50	50	0	50
	96	100	0	100	0	100
	120	100	0	100	0	100
Extract 300 μg/mL	24	100	0	100	0	100
	48	100	0	100	0	100
	72	100	0	100	0	100
	96	100	0	100	0	100
	120	100	0	100	0	100

[a] Percentages relative to the 20 worms investigated.
[b] Control-RPMI 1640 medium.
[c] DMSO-RPMI 1640 medium+0.5% DMSO (solvent control).
[d] PZQ-Praziquantel at 1 μg/mL (positive control).

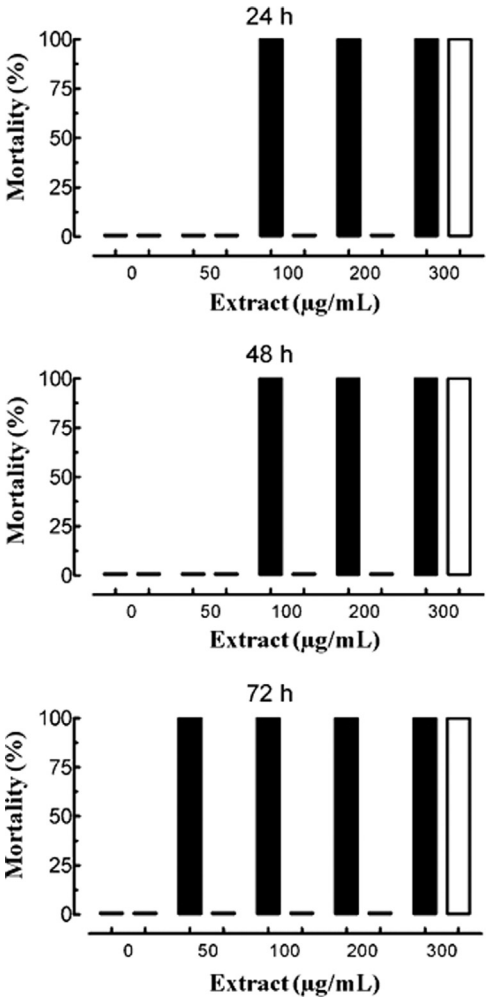

Fig. 4. *In vitro* effects of the Neem EE on the viability of *S. mansoni* adult worms. Pairs of adult worms were exposed to Neem EE at different concentrations during the time periods indicated. Mortality data are presented from ten worm couples. Females (closed bars) and Male (open bars).

mansoni, even because this would be dependent on the mechanisms of action of each agent. However, the fact of the female tegument is slender than the male may be explain the better action of Neem EE due a possible increase in its uptake. This hypothesis should be further evaluated.

To evaluate the effects of Neem EE on the tegument of *S. mansoni*, treated worms were examined using confocal laser scanning microscopy. As shown in Fig. 5A, no abnormality was seen in male worms maintained in culture medium at 120 h of incubation. Meanwhile, in the treatment of the positive control group (1 μg/mL of PZQ) pronounced alterations in the tegument after 24 h were observed (Fig. 5B). The dorsal surface of the tegument of male worms exposed to 300 μg/mL of the extract showed severe morphological changes (Fig. 5C). Tubercles were disintegrated, resulting in the disappearance of buttons and swollen, peeling and surface erosion. With respect to female worms, it was demonstrated that 100 μg/mL of Neem EE caused morphological alterations in the tegument after 24 h (Fig. 6C).

Confocal laser scanning microscopy revealed that Neem EE induced severe tegumental damage in both male and female schistosomes. Similar results were obtained by previous studies using other antischistosomal compounds, such as piplartine, dermaseptin and phytol (Moraes et al., 2011; de Moraes et al., 2011; de Moraes et al., 2014). The schistosomes tegument is an important target for antischistosomal drugs, alterations in the surface topography of worms were used by several studies for the evaluation of antischistosomal drugs activities *in vitro* and *in vivo* (Veras et al., 2012; Guimarães et al., 2014; de Moraes et al., 2014).

These results suggest that Neem EE has antischistosomal activity and encourage us to continue the subsequent experimental and clinical trials. Further studies are needed to evaluate its efficacy against *S. mansoni in vivo*.

4. Conclusion

This work presented two important properties of *A. indica* plant. Its leaf ethanolic extract had the capacity to inhibit MRSA biofilm and planktonic aggregation formation that constitutes important virulence factors of this microorganism. The anthelmintic action of neem against *S. mansoni* worms that cause an important neglected disease, schistosomiasis, was also showed. Further studies to test the constituents of this extract that caused these related effects may result in even more promising results with the use of lower concentrations.

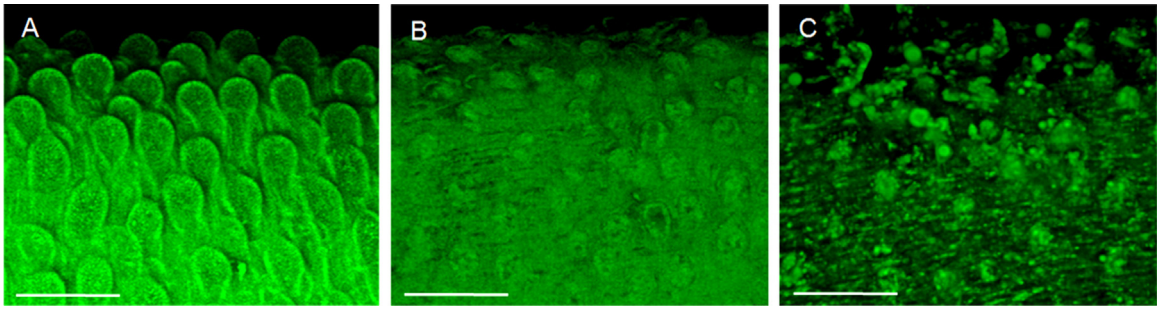

Fig. 5. Confocal laser scanning microscopy investigation of *S. mansoni* male worm after *in vitro* incubation with Neem EE. After 24 h of incubation, adult male worms were fixed in FAA solution and the fluorescent images were obtained using confocal microscopy in dorsal tegumental surface. (A) Negative control (RPMI 1640 medium). (B) Positive control (1 μg/mL praziquantel). (C) Worm treated with 300 μg/mL of Neem EE. Scale bars = 50 μm.

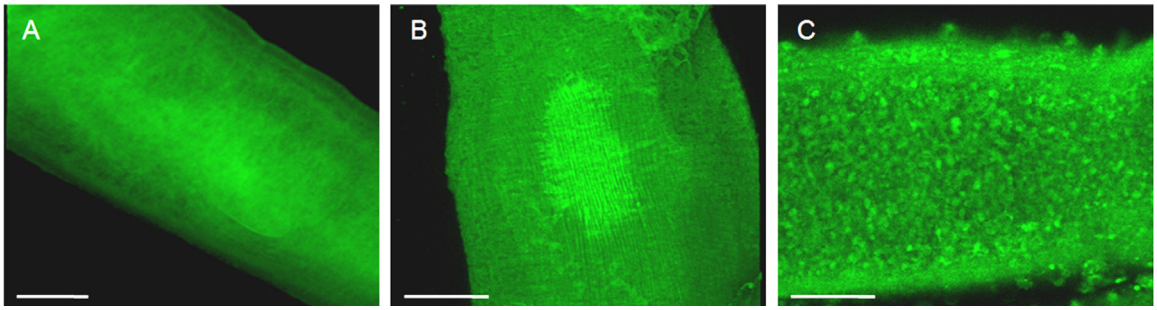

Fig. 6. Confocal laser scanning microscopy investigation of *S. mansoni* female worm after *in vitro* incubation with Neem EE. After 24 h of incubation, adult female worms were fixed in FAA solution and the fluorescent images were obtained using confocal microscopy in dorsal tegumental surface. (A) Negative control (RPMI 1640 medium). (B) Positive control (1 μg/mL praziquantel). (C) Worm treated with 100 μg/mL of Neem EE. Scale bars = 50 μm.

Acknowledgments

The authors are grateful to the received financial support from the projects PP SUS (FAPEPI/CNPq/MS Grant 00045), CNPQ - Ciência Sem Fronteiras/PVE Grant No.400398/2014-1, CNPq/FCT Bilateral Grant 490585/2010-8 and the financial and logistical support for collection and preparation of the extracts from the Phytobios LTDA Company (Centroflora Group).

References

Agyare, C., Spiegler, V., Sarkodie, H., Asase, A., Liebau, E., Hensel, A., 2014. An ethnopharmacological survey and *in vitro* confirmation of the ethnopharmacological use of medicinal plants as anthelmintic remedies in the Ashanti region, in the central part of Ghana. J. Ethnopharmacol. 158, 255–263.

Alzoreky, N.S., Nakahara, K., 2003. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. Int. J. Food Microbiol. 80, 223–230.

Atshan, S.S., Shamsudin, M.N., Sekawi, Z., Lung, L.T.T., Hamat, R.A., Karunanidhi, A., Ali, A.M., Ghaznavi-Rad, E., Ghasemzadeh-Moghaddam, H., Seng, J.S.C., Nathan, J.J., Pei, C.P., 2012. Prevalence of adhesion and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*. J. Biomed. Biotechnol., Article ID 976972, http://dx.doi.org/10.1155/2012/976972 10 pages

Badam, L., Deolankar, R.P., Kulkarni, M.M., Nagsampgi, B.A., Wagh, U.V., 1987. *In vitro* antimalarial activity of neem (*Azadirachta indica* A. Juss) leaf and seed extracts. Indian J. Malariol. 24 (2), 111–117.

Biswas, K., Chattopadhyay, I., Banerjee, R.K., Bandyopadhyay, U., 2002. Biological activities and medicinal properties of neem (*Azadirachta indica*). Curr. Sci. 82 (11), 1336–1345.

Botelho, M.A., Santos, R.A., Martins, J.G., Carvalho, C.O., Paz, C.M., Azenha, C., Ruela, R.S., Queiroz, D.B., Ruela, W.S., Marinho, G., Ruela, F.I., 2008. Efficacy of a mouthrinse based on leaves of the neem tree (*Azadirachta indica*) in the treatment of patients with chronic gingivitis: a double-blind, randomized, controlled trial. J. Med. Plants Res. 2 (11), 341–346.

Chomnawang, M.T., Surassmo, S., Wongsariya, K., Bunyapraphatsara, K., 2009. Antibacterial activity of Thai medicinal plants against methicillin-resistant *Staphylococcus aureus*. Fitoterapia 80, 102–104.

Clinical and Laboratory Standards Institute, 2012. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard, Ninth ed. Clinical and Laboratory Standards Institute, Wayne, PA, USA, CLSI document M07-A9.

Dai, J., Yaylayan, V.A., Raghavan, G.S.V., Paré, J.R., 1999. Extraction and colorimetric determination of azadirachtin-related limonoids in neem seed kernel. J. Agric. Food Chem. 47, 3738–3742.

Donlan, R.M., Corteston, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15 (2), 167–193.

de Moraes, J., Nascimento, C., Miura, L.M., Leite, J.R., Nakano, E., Kawano, T., 2011. Evaluation of the *in vitro* activity of dermaseptin 01, a cationic antimicrobial peptide, against *Schistosoma mansoni*. Chem. Biodivers. 8, 548–558.

de Moraes, J., Nascimento, C., Yamaguchi, L.F., Kato, M.J., Nakano, E., 2012. *Schistosoma mansoni: in vitro* schistosomicidal activity and tegumental alterations induced by piplartine on schistosomula. Exp. Parasitol. 132, 222–227.

de Moraes, J., Carvalho, A.A., Nakano, E., de Almeida, A.A., Marques, T.H., et al., 2013. Anthelmintic activity of carvacryl acetate against *Schistosoma mansoni*. Parasitol. Res. 112, 603–610.

de Moraes, J., Keiser, J., Ingram, K., Nascimento, C., Yamaguchi, L.F., et al., 2013. *In vitro* synergistic interaction between amide piplartine and antimicrobial peptide dermaseptin against *Schistosoma mansoni* schistosomula and adult worms. Curr. Med. Chem. 20, 301–309.

de Moraes, J., de Oliveira, R.N., Costa, J.P., Junior, A.L.G., de Sousa, D.P., Freitas, R.M., Allegretti, S.M., Pinto, P.L.S., 2014. Phytol, a diterpene alcohol from chlorophyll, as a drug against neglected tropical disease *Schistosomiasis mansoni*. PLoS Negl. Trop. Dis. 8 (1), e2617. http://dx.doi.org/10.1371/journal.pntd.0002617.

Doenhoff, M.J., Cioli, D., Utzinger, J., 2008. Praziquantel: mechanism of action, resistance and new derivatives for schistosomiasis. Curr. Opin. Infect. Dis. 21, 659–667.

Fabry, W., Okemo, P.O., Ansorg, R., 1998. Antibacterial activity of East African medicinal plants. J. Ethnopharmacol. 60, 79–84.

Faccin-Galhardi, L.C., Yamamoto, K.A., Ray, S., Ray, B., Linhares, R.E.C., Nozawa, C., 2012. The *in vitro* antiviral property of *Azadiractha indica* polysaccharides for poliovirus. J. Ethnopharmacol. 142, 86–90.

Fallon, P.G., Doenhoff, M.J., 1994. Drug-resistant schistosomiasis: Resistance to praziquantel and oxamniquine induced in Schistosoma mansoni in mice is drug specific. Am. J. Trop. Med. Hyg. 51, 83–88.

Geary, T.G., Klein, R.D., Vanover, L., Bowman, J.W., Thompson, D.P., 1992. The-nervous systems of helminths as targets for drugs. J. Parasitol. 78, 215–230.

Geethashri, A., Manikandan, R., Ravishankar, B., Veena Shetty, A., 2014. Comparative evaluation of biofilm suppression by plant extracts on oral pathogenic bacteria. J. Appl. Pharm. Sci. 4 (03), 20–23.

Guimarães, M.A., Campelo, Y.D.M., Véras, L.M.C., Colhone, M.C., Lima, D.F., Cianciaglini, P., Kuckelhaus, S.S., Lima, F.C.A., de Moraes, J., Leite, J.R.S.A., 2014. Nanopharmaceutical approach of epiisopiloturine alkaloid carried in liposome system: preparation and *in vitro* schistosomicidal activity. J. Nanosci. Nanotechnol. 4 (16), 4519–4528.

Haaber, J., Cohn, M.T., Frees, D., Andersen, T.J., Ingmer, H., 2012. Planktonic aggregates of *Staphylococcus aureus* protect against common antibiotics. PLoS One 7 (7), e41075. http://dx.doi.org/10.1371/journal.pone.0041075.

Hagan, P., Appleton, C.C., Coles, G.C., Kusel, J.R., Tchuem-Tchuente, L.A., 2004.

Schistosomiasis control: keep taking the tablets. Trends Parasitol. 20, 92–97.
Harjai, K., Bala, A., Gupta, R.K., Sharma, R., 2013. Leaf extract of *Azadirachta indica* (Neem): a potential antibiofilm agent for *Pseudomonas aeruginosa*. Pathog. Dis. 69, 62–65.
Hochbaum, A.I., Kolodkin-Gal, I., Foulston, L., Kolter, R., Aizenberg, J., Losick, R., 2011. Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm development. J. Bacteriol. 193 (20), 561–5622.
Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. Antibiotic resistance of bacterial biofilms. Int. J. Antimicrob. Agents 35, 322–332.
Ismail, M., Metwally, A., Farghaly, A., Bruce, J., Tao, L.F., Bennett, J.L., 1996. Characterization of isolates of Schistosoma mansoni from Egyptian villagers that tolerate high doses of praziquantel. Am. J. Trop. Med. Hyg. 55, 214–218.
Jarvis, A.P., Morgan, E.D., Edward, C., 1999. Rapid separation of triterpenoids from neem seed extracts. Phytochem. Anal. 10, 39–43.
Jevons, M.P., 1961. "Celbenin"-resistant staphylococci. Br. Med. J. 1, 124–125.
Kustos, T., Kustos, I., Kilár, F., Rappai, G., Kocsis, B., 2003. Effect of antibiotics on cell surface hydrophobicity of bacteria causing orthopedic wound infections. Chemotherapy 49, 237–242.
Mack, D., Becker, P., Chatterjee, I., Dobinsky, S., Kobloch, J.K.M., Peters, G., Rohde, H., Herrmann, M., 2004. Mechanisms of biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*: functional molecules, regulatory circuits, and adaptive responses. Int. J. Med. Microbiol. 294, 203–212.
Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., 2009. Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J. Bacteriol. 191, 832–843.
Mirani, Z.A., Jamil, N., 2011. Effect of sub-lethal doses of vancomycin and oxacillin on biofilm formation by vancomycin intermediate resistant *Staphylococcus aureus*. J. Basic Microbiol. 51 (2), 191–195.
Mitsui, Y., Miura, M., Aoki, Y., 2009. *In vitro* effects of artesunate on the survival of worm pairs and egg production of *Schistosoma mansoni*. J. Helminthol. 83, 7–11.
Moraes, J., Nascimento, C., Lopes, P.O., Nakano, E., Yamaguchi, L.F., Kato, M.J., Kawano, T., 2011. *Schistosoma mansoni*: *in vitro* schistosomicidal activity of piplartine. Exp. Parasitol. 127, 357–364.
Nicaido, H., 2009. Multidrug resistance in bacteria, 2009. Annu. Rev. Biochem. 78, 119–146.
Okemo, P.O., Mwatha, W.E., Chhabra, S.C., Fabry, W., 2001. The kill kinetics of *Azadirachta indica* a. juss. (meliaceae) extracts on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Afr. J. Sci. Technol. 2 (2), 113–118.
Pai, M.R., Acharya, L.D., Udupa, N., 2004. Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel- a 6-week clinical study. J. Ethnopharmacol. 90, 99–103.
Parida, M.M., Upadhyay, C., Pandya, G., Jana, A.M., 2002. Inhibitory potential of neem (*Azadirachta indica* Juss) leaves on Dengue virus type-2 replication. J. Ethnopharmacol. 79, 273–278.
Pica-Mattoccia, L., Cioli, D., 2004. Sex and stage-related sensitivity of *Schistosoma mansoni* to *in vivo* and *in vitro* praziquantel treatment. Int. J. Parasitol. 34, 527–533.
Polaquini, S.R.B., Svidzinski, T.I.E., Kemmelmeie, R.C., Gasparetto, A., 2006. Effect of aqueous extract from neem (*Azadirachta indica* A. Juss) on *Candida albicans*. Arq. Oral Biol. 51, 482–490.
Ribeiro, A., Dias, C., Silva-Carvalho, M.C., Berquo, L., Ferreira, F.A., Santos, R.N.S., Ferreira-Carvalho, B.T., Figueiredo, A.M., 2005. First report of infection with community-acquired methicillin-resistant *Staphylococcus aureus* in South America. J. Clin. Microbiol. 43 (4), 1985–1988.
Roberts, J.C., 2013. Community-associated methicillin-resistant *Staphylococcus aureus* epidemic clone USA100; more than a nosocomial pathogen. SpringerPlus 2, 133.
Sangster, N.C., Song, J., Demeler, J., 2005. Resistance as a tool for discovering and understanding targets in parasite neuromusculature. Parasitology 131 (Suppl.), S179–S190.
Sharma, V., Walia, S., Kumar, J., Nair, M.G., Parmar, B.S., 2003. An efficient method for the purification and characterization of nematicidal azadirachtins A, B and H, using MPLC and ESIMS. J. Agric. Food Chem. 51, 3966–3972.
Sharma, J., Gairola, S., Sharma, Y.P., Gaur, R.D., 2014. Ethnomedicinal plants used to treat skin diseases by Tharu community of district Udham Singh Nagar, Uttarakhand, India. J. Etnopharmacol. 158, 140–206.
Siddiqui, S., Faiw, S., Siddiqui, B.S., Ghiasuddin, H.E.J., 1992. Constituents of *Azadzrachta indica*: isolation and structure elucidation of a new antibacterial tetranortriterpenoid, mahmoodin, and a new protolimonoid, naheedin. J. Nat. Prod. 55, 303–310.
Sidhu, O.P., Kumar, V., Behl, H.M., 2003. Variability in neem (*Azadirachta indica*) with respect to azadirachtin content. J. Agric. Food Chem. 51, 910–915.
Smithers, S.R., Terry, R.J., 1965. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of adults worms. Parasitology 55, 695–700.
Subapriya, R., Nagini, S., 2005. Medicinal properties of neem leaves: a review. Curr. Med. Chem.-Anticancer Agents 5, 146–149.
Sufian, A.S., Ramasamy, K., Ahmat, N., Zakaria, Z., Yusof, M.I.M., 2013. Isolation and identification of antibacterial and cytotoxic compounds from the leaves of *Muntingia calabura* L. J. Ethnopharmacol. 146, 198–204.
Steinmann, P., Keiser, J., Bos, R., Tanner, M., Utzinger, J., 2006. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect. Dis. 6, 411–425.
Tang, Y.W., Stratton, C.W., 2010. *Staphylococcus aureus*: an old pathogen with new weapons. Clin. Lab. Med. 30, 179–208.
Vasanth, S., Gopal, R.H., Rao, R.H., Rao, R.B., 1990. Plant antimalarial agents. Ind. J. Sci. Res 49, 68–77.
Veitch, G.E., Beckmann, E., Burke, B.J., Boyer, A., Maslen, S.L., Ley, S.V., 2007. Synthesis of azadirachtin: a long but successful journey. Angew. Chem. Int. Ed. Engl. 46, 7629–7632.
Veras, L.M., Guimarães, M.A., Campelo, Y.D., Vieira, M.M., Nascimento, C., Lima, D., Vasconcelos, L., Nakano, E., Kuckelhaus, S., Batista, M.C., Leite, J.R., Moraes, J., 2012. Activity of epiisopiloturine against *Schistosoma mansoni*. Curr. Med. Chem. 19, 2051–2058.
Xiao, J., Zuo, Y., Liu, Y., Li, J., Hao, Y., Zhou, X., 2007. Effects of *Nidus vespae* extract and chemical frations on glucosyltransferases, adherence and biofilm formation of *Streptococcus mutans*. Arch. Oral Biol. 52, 869–875.
Wolinsky, L.E., Mania, S., Nachnani, S., Ling, S., 1996. The Inhibiting effect of aqueous *Azadirachta indica* (neem) extract upon bacterial properties influencing *in vitro* plaque formation. J. Dent. Res. 75, 816–822.
You, Y., Xue, T., Cao, L., Zhao, L., Sun, H., Sun, B., 2014. *Staphylococcus aureus* glucose-induced biofilm accessory proteins, GbaAB, influence biofilm formation in a PIA-dependent manner. Int. J. Med. Microbiol. 304, 603–612.