Original Article

Anti-plasmodial effects of *Azadirachta indica* in experimental cerebral malaria: Apoptosis of cerebellar Purkinje cells of mice as a marker

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Abstract

Background: Malaria is a major public health problem in the world, but treatment of malaria is becoming more difficult due to increasing drug resistance. Therefore, the need for alternative drugs is acute. **Aims:** This study investigated the antiplasmodial and protective effect of an ethanolic extract of the leaves from a traditionally used medicinal plant, *Azadirachta indica* (Neem) in a mouse model of malaria. **Materials and Methods:** Swiss albino mice were intraperitoneally infected with $10x10^6$ *Plasmodium berghei* ANKA, a rodent malaria parasite. The presence of parasites was checked by microscopic examination of blood samples daily. Ethanolic extracts of Neem at 300, 500 and 1000 mg/kg were administered intraperitoneally daily for five days from the day parasitaemia reach 5% of parasite inoculation. Intraperitoneal chloroquine and artemether were used as standard drug treatment controls. Symptoms of neurological or respiratory disorder, mortality, weight and temperature were recorded. Histological sections of brain were prepared and examined after staining with hematoxylin-eosin and immunohistochemistry for apoptotic cells. **Results:** All Neem treatment groups displayed parasitaemia that gradually increased during treatment, and showed signs of terminal illness (i.e. hypothermia, ptosis and convulsions) within 2–4 days post-treatment. In contrast, the chloroquine and artemether groups showed no cerebral malaria symptoms and no deaths. Apoptosis in Purkinje cells, cerebral haemorrhage and oedema were found in some of the mice treated with Neem and chloroquine. **Conclusions:** *Azadirachta indica* (Neem) extract was not protective against malaria symptoms and signs in this mouse model. However, a difference in the number of apoptotic Purkinje cells between the untreated control group and Neem treatment at 500 mg/kg suggests that Neem may have some neuronal protective effect.

Keywords: Experimental cerebral malaria, Neem, antiparasitic, apoptosis, Purkinje cell protection.

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Introduction

Malaria infections are characterized by defective immune responses with poor efficacy against infection, and in some cases, with immunopathology [1]. Severe malaria is a complex multisystem disorder. Complications such as cerebral malaria, anemia, acidosis, jaundice, respiratory distress, renal insufficiency, coagulation anomalies and hyperparasitemia can occur in severe malaria [2]. About 300-500 million clinical cases and 1.2–2.8 million deaths due to malaria occur each year [3, 4]. The rising problem of resistance to the classical drugs (chloroquine and sulphadoxine pyrimethamine) [5-7] and the problem of

recrudescence of malaria after treatment with artemisinin stress the need for new antimalarial agents [8].

The malaria drugs quinine and artemisinin are both plant derivatives and are obtained from *Cinchona* species [9, 10] and *Artemisia annua* [11, 12] respectively, suggesting that other effective malaria drugs might be plant-derived. *Azadirachta indica* plants from the *Meliaceae* family are extensively used as traditional remedies against malaria in the tropics [9, 13, 14]. Several studies demonstrated that *A. indica* leaf, seed and stem bark extracts possess *in vitro* inhibitory activity on *Plasmodium falciparum* asexual stages [15-17]. *In vitro* screening of purified limonoids from Neem revealed that gedunin and nimbolide are the most active molecules against *P. falciparum* [13, 15, 18].

In a previous study, MacKinnon *et al.* examined 60 extracts from 22 species of Meliaceae by characterizing their *in vitro* antiplasmodial activity against chloroquine-sensitive (D6) and chloroquine-resistant (W2) malaria clones (Dd2, Indochina) [15, 19]. Twelve extracts were found to have activity against *P. falciparum*, especially extracts of *Cedrela odorata* wood and *Azadirachta indica* leaves, both containing the limonoid, gedunin [20]. Gedunin has been repeatedly re-isolated as an active antimalarial principle, most recently from *Khaya grandifoliola* [21]. The antimalarial properties of several plant extracts have been studied in mice [22]. Therefore, we sought to examine the anti-plasmodial activity of *Azadirachta indica,* commonly known as Neem, a plant used in Sudan, India and other countries as a folk medicine, in a mouse model of malaria produced in *Plasmodium berghei* ANKA infected mice.

To examine the effects of an antimalarial treatment, we measured physiological and morphological responses. We also examined cerebral malaria. Neuronal ischemia and inflammation from malaria, depending on the acuteness and intensity of the symptoms, can lead to cellular necrosis or apoptosis [23]. Caspases play important roles in the commitment and execution phases of apoptosis [24]. Importantly, caspase-3 acts as a major downstream effector mediating neuronal apoptosis [25, 26]. We examined protection from caspase-dependent apoptosis as a mechanism of malaria treatment effectiveness.

Materials and Methods

Animal care and use
Swiss albino mice weighing 25-30 g and aged 5-7 weeks were donated from The Animal House of the National Health Laboratory, Khartoum, Sudan. The mice were maintained on standard feed and water. Approval for the study was obtained from the Ethical Committee, Institute of Endemic Diseases, University of Khartoum.

Plant collection and sample preparation
The Neem leaves were collected from the state of Khartoum in Sudan, where they are used as a traditional treatment for malaria. The leaves were air dried (in shade) and reduced to powder. The extract was prepared by the maceration of 1500 g of the powdered leaf with 15 liters of ethanol for three consecutive days at room temperature. The extracts were then filtered and concentrated by boiling to produce a final concentration of 1 mg/ml. The extracts were kept in a tightly closed bottle in a refrigerator until used for anti-malarial testing.

Neem extract toxicity test
Toxicity testing was performed with the Neem extracts. Three mice were injected with the extract of the Neem leaves (300, 500 and 1000 mg/kg) intraperitoneally. The mice were acclimatized and fasted over night. Signs of toxicity such as death, changes in physical appearance, behavioral change, and organ damage were recorded for 24 hours.

Parasite
Blood-stage samples of *P. berghei* ANKA were kindly donated by Dr. C.R. Pillai, National Institute of Malaria, New Delhi, India and were maintained in mice. The parasite was conserved as stabilities of 10×10^6 parasitized red blood cells (pRBCs) stored in liquid nitrogen in solution for cryopreservation of the parasite.

Infection of mice
With the exception of the healthy control group, all mice were infected intraperitoneally (ip) with 10×10^6 *P. berghei* ANKA parasitized erythrocytes. The course of infection was followed by a daily determination of parasitaemia by Giemsa-stained blood smear. When the parasitaemia reach approximately 5% of the initial inoculation (at the onset of the cerebral malaria syndrome), treatment groups were assigned. The total number of mice was 35, with 5 mice per group. The first group was not treated (disease control); the second group received chloroquine 12 mg/kg; the third group was given artemether 1.6 mg/kg (dissolved in olive oil); and the fourth group received Neem extract at one of 3 concentrations: 300, 500 or1000 mg/kg (5 mice per concentration). An additional 5 mice served as a healthy control group. All treatments were given ip daily for five days. Parasitaemia, weight, temperature, cerebral symptoms and survival times were recorded for each mouse.

Histopathological analysis
Mice were killed by ether inhalation when they showed signs of terminal illness, i.e., hypothermia, ptosis and convulsions. The brain of each mouse was removed immediately and the cerebellum was placed in 10% neutral buffered formaldehyde. Paraffin-embedded cerebellar tissues were serially sectioned at 3 μm or 5 μm. Cerebellar sections were stained with hematoxylin-eosin (H&E) and an immunohistochemical for active caspase 3.

Neuronal counts
Morphometric analysis was carried out using a light microscope with an eyepiece with a fixed 1 cm grid under 40X magnification to count the number of Purkinje cells in the vermal region of the cerebella of the experimental animals. The cerebella of all 35 mice were serially sectioned, and 5 serial sections of 5 microns thickness were mounted per slide.   The numerical density was obtained by counting

the number of Purkinje cells in 10 fields per section in each cerebellum. The mean and standard deviation of the number of Purkinje cells in each group was calculated.

Immunohistochemistry
Apoptosis in Purkinje cells in the cerebella was determined by immunohistochemistry for caspase activation. Sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and treated with Tris-EDTA buffer pH 9.0 to expose antigenic sites. The sections were treated in 3% H_2O_2 for 10 minutes to inhibit endogenous peroxidase activity. Sections were then incubated with rabbit anti-caspase-3 active antibody (R&D Systems, Minneapolis, MN, US) diluted 1:40 in primary antibody dilution buffer (Diagnostic BioSystems, Serpentine, CA, USA), followed by a biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase (Universal peroxidase kit, Diagnostic BioSystems), prepared according to the kit instructions. The sections were incubated with 3, 30-diaminobenzidine hydrochloride (DAB) chromogen substrate (Diagnostic BioSystems) according to the manufacturer's instructions and counterstained with hematoxylin. Thorough washes between steps were performed using immune wash buffer (Diagnostic BioSystems). Sections were dehydrated through a graded ethanol series, cleared in xylene, and mounted. Sections from tonsil were processed in an identical fashion as a positive control.

Statistical analysis
Data was analyzed using the statistical software SPSS v11.5 (SPSS Inc, Chicago, Illinois, USA). Numerical data were presented as mean ± standard deviation. The significance of the mean difference between two independent groups was determined using Student's t-test, and one-way analysis of variance (ANOVA), or ANOVA with multiple comparisons was used when comparing more than two groups. A *P* value <.05 was considered significant. Proportional data were presented as frequencies and percentages. Significance testing of differences between proportions was conducted using Fisher's exact test. A *P* value <.05 was considered significant unless otherwise stated.

Results

Neem toxicity test
The mice were monitored for 24 hours, but no signs of toxicity were observed. Gross behavioral and physical observations revealed no involuntary urination, muscle weakness, or convulsion. The animals were physically active.

Malaria symptoms and signs
Mice infected with *P. berghei* ANKA became unwell after five days of infection with the following symptoms: ruffled fur; moved slowly or sat hunched; locomotors disturbances with paresis; convulsions or ptosis (drooping of upper eye lids); and respiratory disturbance.

Symptoms and signs of cerebral malaria (ptosis, ruffled hair, respiratory stress and paralysis) were observed in all

Neem extract treated groups on day 3 of treatment. All of the mice were sacrificed when they showed sign of terminal illness. However, the group treated with 500 mg/kg Neem showed high physical activity, high parasitaemia (53%), normal temperature, weight and number of Purkinje cells, i.e., no neuronal apoptosis.

The deaths in the disease control group started on day 3 of treatment, i.e., day 8 of inoculation with *P berghei* ANKA. Fifty percent of the disease control group were dead by day 3 of treatment, and the number of deaths increased to 100% by day 5 of treatment. Forty-six percent of the animals in the Neem treatment groups died by day 2 of the treatment. The percentage of animals dying in the Neem treatment groups increased to 86.7% by day 4 when animals showed signs of terminal illness, i.e., hypothermia, ptosis and convulsions. No deaths were seen in the chloroquine or artemether treatment groups by day 4 of the treatment.

At the end of the study, i.e., day five of treatment, 18/33 (54.5%) of the mice infected were still alive. All mice were sacrificed at the end of the study.

The results of the study indicated that alcoholic extracts of *A. indica* displayed no activity against the *P. berghei* ANKA malaria parasite (Fig.1).

All of the mice treated with ethanol extracts of Neem (300, 500 and 1000 mg/kg) displayed increased parasitaemia gradually from day 0 of treatment (0 5%, 5.1% and 7.2% respectively). The peak levels occurred at day 4, with a mean parasitaemia of 53% by day 4 of treatment, and a decline to 21.9% by day 3 of treatment.
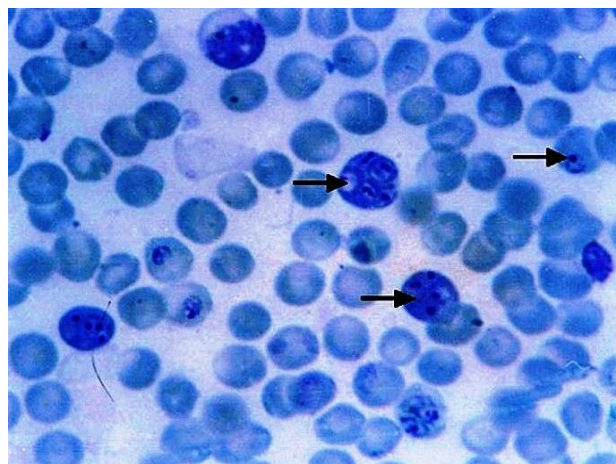
Fig. 1 Photomicrograph showing parasitemia (arrow) in mice blood infected with *P. berghei ANKA*, Geimsa Stain X 1000.

The untreated disease control mice displayed increased parasitaemia from day 0 of treatment (5%) to day 4 of treatment (9.9%). The chloroquine and artemether treatment groups displayed decreased parasitaemia gradually from day 0 of treatment (3.9% and 3.6% respectively) to day 4 of treatment (0.5% and 0.3% respectively). The parasitaemia was cleared to 0% in the

chloroquine and artemether groups by day 6 of treatment. There was no significant difference in the mean parasitaemia levels between the chloroquine and artemether groups at day 0 and day 5 of treatment (Figure 2). There was no significant difference between day 0 of treatment parasitaemia in the study groups (Tables 1-3).
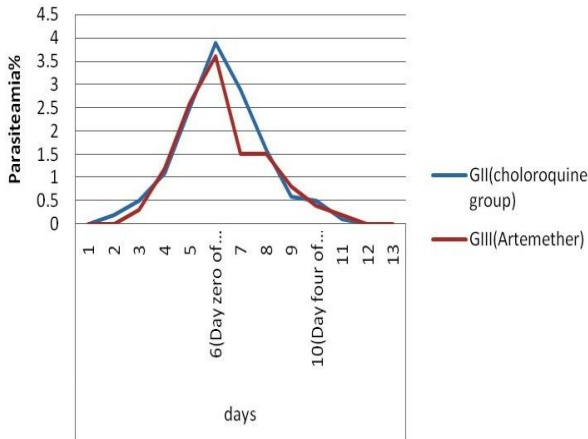
Fig. 2 Chart showing comparison of mean parasitaemia among GII : chloroquine treated group and GIII: artemether treated group. No significant difference was found between the 2 groups.

Table 1 Mice parasitaemia of 300 mg/kg Neem compared to control groups

Day of treatment	Disease control	Chloroquine	Artemether	Neem 300 mg/kg
Day 0	4.6%	3.9%	3.6%	5%
Day 3	6.2%	1.5%*	0.9%*	10%
Day 4	9.9%**	0.5%*	0.3%*	22.8%

* Significantly different (*P*<.05), ** Statistical test was not performed; only one experimental mouse remained alive in each group.

Table 2 Mice parasitaemia at 500 mg/kg Neem compared to control groups

Day of treatment	Disease control	Chloroquine	Artemether	Neem 500 mg/kg
Day 0	4.6%	3.9%	3.6%	5.1%
Day 3	6.2%	1.5%	0.9%	13.8%
Day 4	7.3%**	0.6%*	0.5%*	53%

* Significantly different (*P*<.001), ** Statistical test was not performed; only one experimental mouse remained alive in each group.

Table 3 Mice parasitaemia at Neem 1000 mg/kg compared to other control groups

Day of treatment	Disease control	Chloroquine	Artemether	Neem 1000 mg/kg
Day 0	4.6%	3.9%	3.6%	7.2%
Day 3	6.2%	1.5%*	0.9%*	21.9%

* Significantly different (*P*<.05).

Weight
There was no significant difference between groups in weight at day 0 of infection and the last day of treatment.

Temperature
There was no significant difference in rectal temperature between the disease control and Neem groups at day 3 of treatment.

There was a significant difference between the disease control compared to the chloroquine and artemether groups (*P*=.038 and .032) at day 4 of treatment.
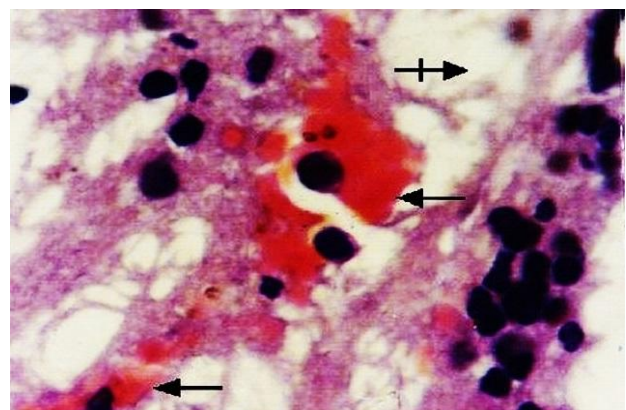
Cerebellar neuronal apoptosis and number of Purkinje cells
There was no significant difference between healthy control and artemether groups (Table 4).

The percent of Purkinje cells displaying apoptosis was: disease control = 20%, chloroquine = 25%, Neem 300 mg/kg = 60%, Neem 500mg/kg = 25% and Neem1000mg/kg = 33%. There was no significant difference between the different experimental groups at postmortem with respect to neuronal apoptosis (Figure 3).
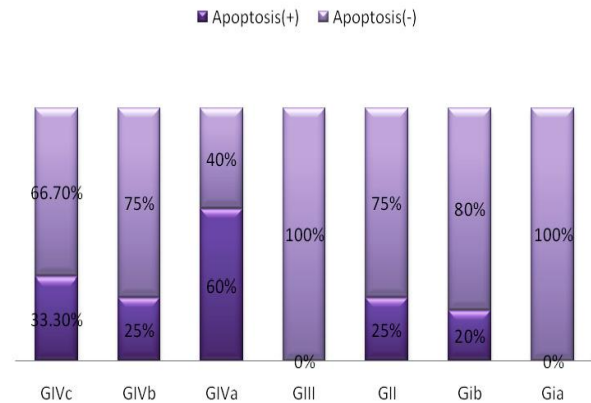
Fig. 3 Chart showing comparison of Purkinje cells apoptosis among the experimental groups. No significant difference was found between groups. G Ia: No infection control group, G Ib: Disease control group, G II : Chloroquine treated group, GIII: Artemether treated group, GIVa: Neem 300mg/kg treated group, GIVb: Neem 500mg/kg treated group, GIVc: Neem 1000mg/kg treated group.(+): present, (-) absent.

Fig. 4 Photomicrograph showing hemorrhage (arrow), and edema (double arrow), in cerebellum H&E stain, x 400.

No significant difference in the number of Purkinje cells displaying apoptosis was found between the disease control and any Neem group.

Hemorrhage

Cerebellar hemorrhages were seen with a frequency of 25% in the disease control and Neem 500 mg/kg groups); 40% in the Neem300mg/kg group; and 33% in the Neem 1000 mg/kg group. No hemorrhages were seen in the healthy control, chloroquine, or artemether groups (Figs. 4, 5). There was no significant difference in cerebellar hemorrhage between the disease control and Neem groups.

Cerebellar edema

Cerebellar edema was seen with a frequency of 40% in the disease control group; 60% in the Neem 300 mg/kg group; 25% in the Neem 500 mg/kg group; and 67% in the Neem 1000 mg/kg group. No edema was seen in the healthy negative control group, or chloroquine or artemether groups (Figs. 6AB, Fig. 7). There was no significant difference in cerebellar edema between the disease control and Neem groups.

Table 4 Mean ± standard deviation of the number of Purkinje cells in the healthy control group compared to other study groups.
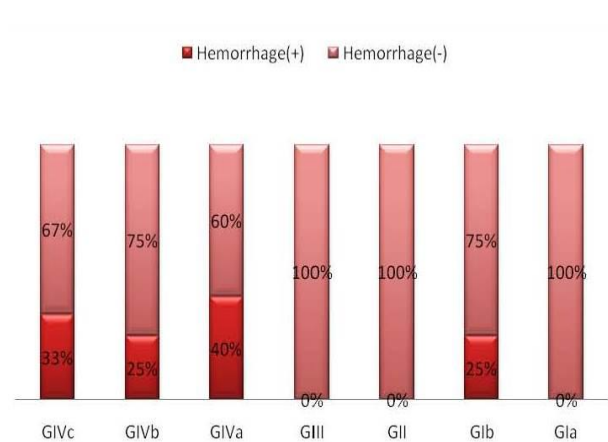
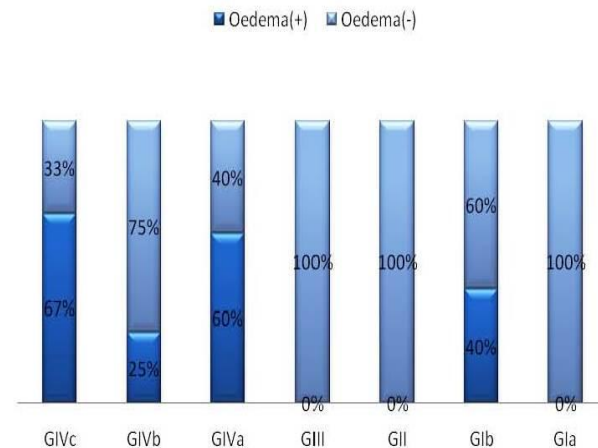|	Healthy control	Disease control	Chloroquine	Artemether	Neem mg/kg		
					300	500	1000
Mean ±SD	8.2 ± 1.0	6.8 ± 0.4*	6.0 ± 0.9 *	7.2 ±1.1	5.7 ± 0.4*	7.2 ± 0.5*	8.2 ± 1.4*

* Significantly different (*P*<.05)

Table 5 Number of Purkinje cells in disease control group compared to other groups

|	Disease control	Chloroquine	Artemether	Neem mg/kg		
				300	500	1000
Mean ± SD	6.8 ± 0.4	6.0 ± 0.9	7.2 ± 1.1	5.7 ± 0.4*	7.2 ± 0.5	8.2 ± 1.4

* Significantly different (*P*<.05).

Fig. 5 Chart showing the number of mice that showed cerebellar hemorrhage in different treatment groups. G Ia: No infection control group, G Ib: Disease control group, G II : Chloroquine treated group, GIII: Artemether treated group, GIVa: Neem 300mg/kg treated group, GIVb: Neem 500mg/kg treated group, GIVc: Neem 1000mg/kg treated group. (+): present, (-) absent.

Fig. 7 Chart showing the number of mice that showed cerebral edema in different groups. GIa: No infection control group, G Ib: Disease control group, G II : Chloroquine treated group, GIII: Artemether treated group, GIVa: Neem 300mg/kg treated group, GIVb: Neem 500mg/kg treated group, GIVc: Neem 1000mg/kg treated group. (+): present, (-) absent.
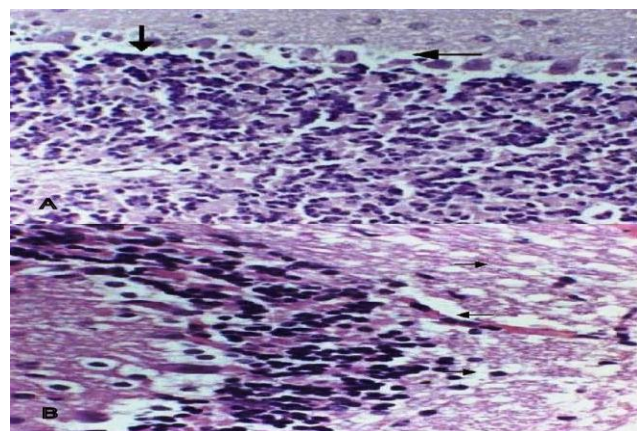
Fig 6 (a) Photomicrograph showing oedema (arrow), in Purkinje cell layer of cerebellum in mouse infected with malaria H&E stain, x 160. (**b**) Photomicrograph showing oedema (arrow), in cerebellum in mouse infected with malaria H&E stain, x 1000.

Discussion

Cerebral malaria is an immune-physiopathologic process caused by *Plasmodium falciparum* in humans and *Plasmodium berghei* ANKA in rodents. The symptoms associated with cerebral malaria in mouse models include respiratory distress syndrome, decreased body temperature, and neurological manifestations characterized by ataxia, paralysis (mono-, hemi-, and tetraplegia), and coma, followed by death [27]. The histopathological changes involved in both human and murine cerebral malaria are characterized by loss of vascular cell integrity, tissue edema [28], hemorrhages in the brain of mice [29-31] and congestion of micro-vessels with parasitized erythrocytes and/or mononuclear cells [28]. The antimalarial activity of the Neem has been attributed to the presence of β-sitosterol [32], nimbolide [33], gedunin and other

limonoids [20].

Results of this study indicated that in this mouse model, ethanolic extracts of Neem leaves administered at 300, 500 and 1000 mg/kg displayed no activity against the *P. berghei* ANKA malaria parasite, and the extract was not effective in preventing death due to cerebral malaria during treatment. These results agree with tests using Neem extracts in malaria-infected mice and chicks that were uniformly disappointing [34]. This is despite the fact that an ethanolic extract of Neem leaves had an IC_{50} value of 5 µg/ml against *P. falciparum* in culture [35]. The same authors also reported that Neem ethanolic extract and aqueous decoctions did not show any activity in a 4 day test against *P. berghei* in mice administered either orally or sub-cutaneously at levels up to 746 mg/kg/day [35]. The same results were obtained with gedunin/Neem leaf and bark when administered to *P. berghei/ P. gallinaceum*-infected mice [18, 36]. However, it was also shown that large doses of an aqueous decoction of Neem leaves reached 90% effective dose (23,782 mg/Kg) in *P. berghei*-infected mice [37].

On the other hand, the *in vitro* anti-malarial activity of Neem was previously reported by El-Tahir *et al* in 1999, where they reported that an aqueous extract of Neem leaves showed an IC_{50} value <5 µg/ml against *P. falciparum* [38]. Similar results were obtained by Alshawsh and colleagues in 2007, when they reported that aqueous Neem extracts inhibited the development of the ring stage of *P. falciparum* [39].

The dilemmas with rodent models for malaria are many: in humans, the causative agents of malaria are *Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae,* and *Plasmodium vivax,* none of which cause infection in rodents. Similarly, *Plasmodium berghei* ANKA, which was used in this study, causes malaria only in rodents but not in humans. It is also well documented that trimethoprim is ineffective against murine malaria, but effective in humans. These findings probably indicate that *P. berghei* has different properties and sensitivities from *P. falciparum.* Finally, the plant extracts are only administered to mice for 4 days. Neem extracts may have a different pharmacokinetic profile from chloroquine and artemether, requiring more frequent doses for longer periods to maintain therapeutic plasma concentrations.

The effect on parasitaemia of leaf extract of Neem was not dose dependent as was shown by Obaski and Jegede-Fadunsin in 1986 [40]. The efficacy peaked 200 mg/kg and declined at higher doses.

In this study, the Neem extract failed to prevent the reduction in body temperature, ptosis, convulsions and increased parasitaemia/ These results are in agreement with Dikasso *et al*.[41] and Bagot *et al*.[42]. All mice in the Neem treatment groups showed symptoms and signs of disease and died, except one mouse, which showed high activity, high parasitaemia (53%), normal temperature, normal weight, and normal number of Purkinje cells and died 10 days later from anemia due to hyperparasitemia. This variation may be similar to adults that show resistance, and to the many asymptomatic individuals with immunity to malaria based on exposure to *Plasmodium* antigen [43].

A previous studies showed that antimalarial activities of a tablet suspension of the bark and leaf of Neem exhibited high prophylactic, moderate suppressive, and a very minimal curative schizonticidal effect in experimental mice [44]. This study found alcoholic extract of Neem failed in this study to significantly prevent hemorrhages and edema in the cerebellum of *P. berghei* ANKA-infected mice. This is in total agreement with previous studies [45-48].

Neuropathological studies have paid little attention to the mechanisms of cell death in the course of cerebral malaria in experimental animals. Besides necrosis, apoptosis has been demonstrated as an important contributor to neuronal and glial damage and pathogenesis in various acute neurological diseases like cerebral ischemia, bacterial meningitis and head injury [26, 49, 50]. In this study, apoptosis (immune-reactivity for caspase-3) was detected in the cerebellum in all groups of mice with clinical signs of cerebral malaria except the artemether group. The extent of apoptosis was correlated to the clinical severity of the disease. Neem extract failed significantly to prevent apoptosis. This result is in agreement with the findings of Lackner *et al*. [51], that showed widespread activation of caspase-3 reactivity in the neurons, oligodendrocytes and endothelial cells of *P. berghei* ANKA-infected mice.

The number of Purkinje cells in the 500 mg/kg and the 1000 mg/kg Neem groups was similar to their number in the healthy control and the artemether groups. This possible chemoprevention of cell loss by Neem leaf extract may be associated with cell proliferation but without an effect on the induction of apoptosis [52].

Conclusion

Ethanolic extracts of *Azadirachta indica* (Neem) displayed no activity against the *P. berghei* ANKA malaria parasite. Edema and hemorrhage were worse in the 300 mg/kg and 1000 mg/kg Neem treatment groups, while chloroquine and artemether showed no hemorrhage and edema. Neem extract was not effective in preventing apoptosis due to cerebral malaria during treatment. However, unlike chloroquine, the high dose levels of Neem extract (500 mg/kg and 1000 mg/kg) prevented Purkinje cells loss. This specific effect may deserve further studies.

Acknowledgement

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