Azadirachta indica (Neem) extract as natural agent against experimental toxoplasmosis

Hoda A Rashed¹, Salwa S Younis³, Amany Abdel Bary², Eman A Elmorsy¹

Departments of Medical Parasitology¹ and Pathology², Faculty of Medicine, Alexandria University, Egypt

ABSTRACT

Background: Toxoplasmosis treatment with the current gold standard combination of pyrimethamine and sulfadiazine or triple sulphonamides, is associated with adverse side effects. There is an urgent need to investigate new natural products with high efficacy and minimum side effects.

Objective: To evaluate the therapeutic potential of neem oil against experimental acute toxoplasmosis.

Material and Methods: Four hours after infection with T. gondii (RH strain) tachyzoites, mice were treated with neem oil extract, and its efficacy was compared versus Septrin (trimethoprim-sulfamethoxazole). Parasitological evaluation included tachyzoite counts in the peritoneal fluid, splenic and hepatic tissue, as well as survival time. Tachyzoites ultrastructural alterations were recorded using both scanning and transmission electron microscope (SEM, and TEM). Immunological study was also conducted by assessment of serum IFN-γ level.

Results: Treatment improved the outcome of infection as the mice survival time reached 9.7±1.4 days with reduction in tachyzoites load and viability reaching 77.6% and 44.4%, respectively. Histopathological examination of hepatic and splenic tissue sections showed less lymphocyte infiltration, less necrosis with scanty free tachyzoite in the section. Compared to Septrin-treated mice, IFN-γ level showed significant rise.

Conclusion: Neem oil extract is a potential therapeutic agent against acute toxoplasmosis.

Keywords: experimental acute toxoplasmosis; IFN-γ, neem oil.

Received: 7 November, 2023; Accepted: 28 December, 2023.

Corresponding Author: Eman A. Elmorsy; Tel.: +20 12 2717 4080; Email: eman.elmorsy@alexmed.edu.eg


INTRODUCTION

Toxoplasma is one of the apicomplexans that infects about one third of the human population[1]. Human acquires infection by ingestion of mature sporulated oocysts contaminating food or water. Other source of infection is consumption of imperfectly cooked meat containing the Toxoplasma cyst[2]. So long as its host is immune competent, toxoplasmosis passes unnoticed or with mild flu like manifestations. However, toxoplasmosis could be a life-threatening condition in immunosuppressed hosts causing severe neurological and ocular manifestation. Additionally, active toxoplasmosis during pregnancy could result in different outcomes that vary from mild congenital anomalies up to still birth and abortion[2,3].

Nowadays, the therapeutic role of several plants was investigated as they have the advantage of minimal or no side effects and are affordable. A study investigated the therapeutic efficacy of 12 different extracts from wild Egyptian plants against T. gondii in vitro. Four plant extracts; Ritemista judaica, Cleome droserifolia, Trichodesma africanaum, and Vachellia tortillis showed promising results in reducing parasite replication with no significant toxicity[4]. Compared to Spiramycin, curcumin nanoemulsion showed similar results, and the investigators recommended its use since it improved the outcome of acute toxoplasmosis without side effects[5].

Among the natural products that gained medical attention is the neem tree (Azadirachta indica) that is commonly cultivated in the southern regions of Africa and Asia. Notably, its components were used for many years as traditional remedy. It contains several bioactive components such as nimbin, nimbidin, nimboline, limonoids, and flavonoids[6]. Regarding the biological activities of neem oil, it has free radical scavenging properties and is considered a rich source of antioxidants such as azadirachtin and nimboline[7]. Later, Hossain et al.[8] investigated its chemical components in Omani neem leaves using different extraction methods. The study showed that it has high level of antioxidant activity due to its phenolic compounds, alkaloids, terpenoids and their derivatives. This highlights the possibility of using crude extract of neem tree as a natural source of antioxidant[8].

Recently, a study proposed it as a cost-effective mean to deliver an antioxidant with minimal side effects and low toxicity[9]. It also showed anti-
inflammatory activity by modulating proinflammatory enzyme activities such as cyclooxygenase, and lipoxygenase.\(^\text{[9]}\) Besides, it exhibited modulatory roles in the gene involved in apoptosis through B-cell lymphoma family genes.\(^\text{[10,11]}\)

Several studies were conducted to verify neem beneficial effects against variable medical diseases such as diabetes mellitus type II\(^\text{[12]}\); mammary carcinogenesis\(^\text{[13]}\); periodontal disease\(^\text{[14]}\); and cancer prostate\(^\text{[15]}\). Its promising results as antimicrobial, analgesic and antipyretic agent were reported\(^\text{[16]}\). Neem oil effects were also studied for protection against ectoparasites. It showed more than 70% protection when used as repellent against Anopheles arabiensis bite\(^\text{[17]}\), and exhibited significant improvement when combined with coconut oil for topical treatment of tungiasis\(^\text{[18]}\). In a recent study, the investigators used neem-silver nitrate nanoparticle that showed potent antimalarial activity with four folds reduction in its half inhibitory concentration in comparison to aqueous neem extract\(^\text{[19]}\).

Although being investigated against various medical conditions, and parasitic diseases, neem oil was not investigated against experimental toxoplasmosis. This encouraged us to investigate its role as a potential therapeutic agent against experimental acute toxoplasmosis.

**MATERIAL AND METHODS**

This case-control study was conducted at the Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt during the period from April 2023 to September 2023.

**Study design:** Swiss albino mice were infected with *T. gondii* RH strain. The therapeutic effect of neem oil was assessed in comparison to Septrin using parasitological, ultrastructural, histopathological, and immunological tests. To investigate its safety, a biochemical study was conducted to determine the possibility of inducing hepatic and/or renal toxicity.

**Parasitic strain:** The *T. gondii* RH virulent strain was utilized in this study. It was propagated in our laboratory through serial intraperitoneal (IP) injection of 2500 tachyzoites. Five days post infection (PI), peritoneal cavity lavage was performed for collection of tachyzoites in phosphate buffer saline (PBS). To remove peritoneal cells and debris, harvested tachyzoites were centrifuged at low speed of 100 ×g for 10 min at 4°C.\(^\text{[19]}\)

**Experimental animals:** Forty-eight free laboratory-bred male Swiss albino mice aged 3-5 w and weighing 20-25 g were used. They were housed in separate cages, in a well-ventilated room in the animal house of the Medical Parasitology Department, Faculty of Medicine, Alexandria University. They were fed commercial diet formed of fibres and proteins and allowed water ad libitum. Bedding was changed daily.

**Drugs and administration regimens:** Septrin (GlaxoSmithKline) was used as a therapeutic control. The oral suspension formulation (40 mg trimethoprim plus 200 mg sulphamethoxazole) was purchased from a local pharmacy. It was given at a dose of 0.5 mg/kg/day. This was prepared as five ml of the Septrin oral suspension added to 995 ml PBS, pH 7.4, to obtain a final drug concentration of 240 mg/l from which 50 μl/mouse/day were administered. Herbal apothecary neem oil 100% pure, certified organic, and cold pressed to preserve natural active ingredients, was purchased from online markets in 50 ml bottle volume. Neem oil was given at a dose of 5 ml/kg/day. All drug formulations were administered orally by gavage needle for five days starting 4 h PI.\(^\text{[20]}\)

**Study groups:** The study included two groups: non infected group (GI, n.o.=12), infected group (GII, n.o.=36). In group I, mice were divided equally into two subgroups; Ia representing the negative control subgroup, and subgroup Ib orally inoculated with neem oil to assess its safety. The infected GII comprised three equal subgroups. Subgroup Ila specified as the infected non treated control, subgroup Iib represented the therapeutic control that received Septrin, and subgroup Ilc represented the experimental mice that received neem oil.

**Mice infection and sacrifice:** Mice were infected through IP inoculation of 2500 tachyzoites of virulent RH strain.\(^\text{[20]}\) Six mice from each infected subgroup were sacrificed on the 6th day PI to assess drug efficacy. The remaining six mice in each subgroup were observed daily until all experimental mice died, i.e., three weeks PI to estimate the survival time.

**Blood samples and specimens’ collection:** Blood samples (2 ml) were collected by cervical incision before euthanasia. Coagulated blood samples at room temperature were centrifuged at ~2000 ×g for 20 min. Serum samples were aliquoted and stored at -20°C for INF-γ assay and biosafety biochemical study. The peritoneal fluid collected by peritoneal cavity lavage was freshly examined for tachyzoite count. A drop was fixed on a slide and stained by trypan blue for tachyzoites viability, and an aliquot was preserved in 2.5% glutaraldehyde for tachyzoites ultrastructure examination. Livers and spleens were collected, and each was divided into two equal parts, one for performing impression smear, and the other part was preserved in 10% formalin for histopathological examination.\(^\text{[20]}\)

**Survival time:** Daily recording of mice mortality to estimate survival time until death of all infected mice (6 mice in each subgroup)\(^\text{[20]}\).
Neem oil against acute toxoplasmosis

Tachyzoites count: Using a haemocytometer, *T. gondii* tachyzoites per ml of the peritoneal fluid of each infected mouse were counted, and the mean count±SD of each subgroup was calculated. The mean tachyzoite count±SD in Giemsa-stained impression smears of liver and spleen of infected mice subgroups was also calculated. For each mouse, the extracellular tachyzoites were counted in 10 different oil lens fields then the mean number was estimated and followed by recording the mean number of all mice in each subgroup\(^{(19)}\). Parasite burden reduction was calculated according to the following formula: Percentage reduction (%R) = 100 − [(n ± N) × 100], where (n) is the mean value in each subgroup and (N) is the mean value of the control subgroup\(^{(21)}\).

Parasite viability: Tachyzoites obtained from the peritoneal fluid of each *Toxoplasma* infected mouse was separately stained with 0.4% trypan blue to test their viability. Viability rate was calculated for each mouse by counting the viable tachyzoites per 100 parasites using the high-power lens (X400). The step was repeated thrice, and the mean viability rate was determined for each group\(^{(21)}\).

Morphological study: On the day of sacrifice (6\(^{th}\) day), peritoneal exudate collected from infected non treated and neem treated and Septrin treated subgroups were fixed in 2.5% glutaraldehyde and prepared for further examination of tachyzoites ultrastructure by SEM and TEM\(^{(19)}\).

Immunological and biochemical studies: The level of INF-γ was measured in the sera of all studied subgroups by ELISA kits (Biospes Co., Ltd) according to the manufacturer instructions\(^{(22)}\). Sera from subgroup Ib were used to demonstrate the safety of neem oil on liver and kidney functions. Both aspartate transaminase (AST) and alanine transaminase (ALT) were measured as marker for hepatic cell integrity, while urea and creatinine were assayed for kidney function\(^{(22)}\).

Histopathological study: To assess histopathological changes, specimens from the liver and the spleen of all infected GII subgroups were fixed in 10% of neutral formalin, sectioned and stained with H&E stain\(^{(22)}\).

Statistical analysis: Data were recorded and analysed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). For continuous data, they were tested for normality by the Shapiro-Wilk test. Quantitative data were expressed as range (minimum and maximum), mean±SD. One way ANOVA test was used to compare the three studied groups and followed by Post Hoc test (Tukey) for pairwise comparison. Significance of the obtained results was judged at the 5% level (P<0.05).

Ethical considerations: This study was conducted after approval by the Ethics Committee of the Faculty of Medicine, Alexandria University (00012098) following the international regulations of laboratory animal care.

RESULTS

Survival time: As shown in Kaplan Meier survival curve for all infected GII subgroups, the maximal survival of infected non treated subgroup (IIa) was 8 days with a mean of 5.83±1.5 days. Compared to infected non treated subgroup, both Septrin treated (IIb) and neem oil treated (IIc) subgroups showed a statistically significant prolongation of mice survival up to 14 and 12 days, with means of 10.3±2.3, and 9.7±1.4 days, respectively (Fig. 1). Interestingly, there was a statistically non-significant difference in mice survival between both subgroups.

Tachyzoite count: As shown in figure (2), there was a statistically significant reduction in mean tachyzoite count in peritoneal fluid and organ impression smears in both treated subgroups (IIb, IIc) compared to infected non treated subgroup (IIa) (P<0.001). There was a statistically significant difference in favour of Septrin compared to neem oil in peritoneal fluid parasitic load reduction (94.6% versus 77.6% reduction, respectively). Tachyzoite count in organ impression smears, indicated that mice subgroup treated with neem oil (IIc) yielded...
a reduction of 76.1%, and 85.8% in hepatic, and splenic impressions, respectively. Favourably, these reductions were statistically non-significant compared to those recorded in Septrin treated subgroup (IIb) (81.8%, and 86.6%, respectively).

Parasite viability: A statistically significant reduction in tachyzoites viability was achieved in both treated subgroups (IIb, IIc) (Fig. 3). Compared to infected non treated subgroup (IIa), the mean percentage of viable parasites retrieved from neem treated subgroup (IIc) was 55.2±7.3 with a reduction of 44.4%, while the result for Septrin-treated subgroup (IIb) was 37.5±5.3 with a reduction of 62.2%. Additionally, Septrin-treated subgroup showed a statistically significant difference in viable parasite percentage reduction in comparison to neem treated subgroup (P<0.001).

Morphological results: In SEM, tachyzoites gathered from peritoneal fluid of infected non treated subgroup (IIa) showed completely regular and intact outline with smooth surfaces as well as prominent conoid (Fig. 4A). Those of neem oil treated subgroup (IIc) showed severe morphological deformities in the form of swelling and ballooning of most parasites associated with wall disruption (Fig. 4C and D). Some of these tachyzoites exhibited severe degenerative changes with shrunken size (Fig. 4E). These changes were similar to those observed in the tachyzoite obtained from Septrin-treated mice (IIb) (Fig. 4B). In TEM examination, tachyzoites of infected non treated subgroup (IIa) revealed smooth intact cellular membranes of the crescent shaped tachyzoites. Prominent secretory rhoptries (Fig. 5A) and dense granules (Fig. 5B) were apparent. Evident nucleus with intact nuclear membranes and prominent nucleolus was shown in transverse cut section (Fig. 5B). In contrast, tachyzoites of neem oil treated subgroup (IIc) showed extensive vacuolations of the cytoplasm (Fig. 5D) and undistinguishable internal structures with wall irregularities (Fig. 5E). Tachyzoites obtained from Septrin-treated subgroup (IIb) showed also extensive vacuolation of the cytoplasm with irregular outline and distorted morphology (Fig. 5C).

Immunological and biochemical results: Immunologically, neem oil administration to mice of subgroup (Ib) induced a statistically non-significant increase in the mean INF-γ concentration compared to non-infected non-treated subgroup (Ia). All infected
subgroups (Ia, Ib and Ic) showed a statistically significant increase in the mean INF-γ concentration compared to non-infected subgroups (la and lb) \((P<0.001)\) (Fig. 6). Compared to the control positive infected non-treated subgroup (Ia), neem oil treated subgroup (Ic) showed a statistically significant increase in the mean INF-γ concentration \((P<0.001)\). In contrast, Septrin treated subgroup (Ib) recorded a statistically non-significant increase in the mean INF-γ concentration. A statistically significant difference in the mean INF-γ concentration was observed between treated subgroups with neem oil and Septrin in favour of neem oil \((P<0.001)\).

Biochemically, a statistically non-significant difference in the mean level of hepatic transaminases and alkaline phosphatase was observed in neem oil administered non-infected subgroup (lb) compared to la. Similarly, a statistically non-significant difference in the mean level of blood urea and serum creatinine was recorded in both subgroups (Table 1).

### Table 1. Biochemical liver and kidney function tests (neem biosafety) of non-infected non-treated subgroup (la) and non-infected neem treated subgroup (lb).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal non-infected (la) (No. = 6)</th>
<th>Non-infected + Neem (lb) (No. = 6)</th>
<th>Statistical analysis</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Min. – Max. Mean ± SD</td>
<td>Min. – Max. Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>55.2 – 60.1 58.18 ± 1.8</td>
<td>56.7 – 62.6 59.28 ± 2.2</td>
<td></td>
<td>0.374</td>
</tr>
<tr>
<td>AST</td>
<td>Min. – Max. Mean ± SD</td>
<td>Min. – Max. Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>240.7 – 301.4 266.5 ± 23.2</td>
<td>267.9 – 289.5 279.3 ± 8.6</td>
<td></td>
<td>0.255</td>
</tr>
<tr>
<td>ALP</td>
<td>Min. – Max. Mean ± SD</td>
<td>Min. – Max. Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>155 – 185 172.7 ± 10.7</td>
<td>167 – 197 184.7 ± 4.3</td>
<td></td>
<td>0.079</td>
</tr>
<tr>
<td>BUN</td>
<td>Min. – Max. Mean ± SD</td>
<td>Min. – Max. Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.4 – 11.4 10.3 ± 0.69</td>
<td>10.1 – 11.4 10.7 ± 0.51</td>
<td></td>
<td>0.310</td>
</tr>
<tr>
<td>CRE</td>
<td>Min. – Max. Mean ± SD</td>
<td>Min. – Max. Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.6 – 37.4 33.4 ± 2.3</td>
<td>32.5 – 37.4 35.5 ± 2.1</td>
<td></td>
<td>0.132</td>
</tr>
</tbody>
</table>

### Histopathological results: The H&E-stained liver tissue sections of infected non treated control subgroup (Ia) scarified 6 days PI displayed mild distortion of the liver architecture with loss of hepatocytes cords continuity and generalized infiltration of inflammatory cells and Kupffer cell hyperplasia loaded with many tachyzoites besides free tachyzoites. This was associated with severe congested expanded central vein and peri-central moderate lymphocytic infiltration. The hepatocytes showed varying degree of fatty changes and formation of pseudocysts containing tachyzoites, and multiple foci of necrosis associated with fibrosis and expansions (Figs. 7A and B). On the contrary, H&E-stained liver tissue sections of infected treated subgroups displayed amelioration of pathological changes. In Septrin treated subgroup (Ib) a preserved liver architecture was evident with mild peri-portal lymphocytic infiltration associated with Kupffer cell hyperplasia loaded with few tachyzoites and no free tachyzoites. Moderate vascular congestion of expanded central vein was also noted with no demonstrated necrotic foci (Figs. 7 C and D). Similarly, in neem oil treated subgroup (Ic), mild congested central veins associated with a very mild peri-central lymphocytic infiltration and no free tachyzoites was noted in a preserved liver parenchymal architecture. Scanty small pseudocysts with few tachyzoites were detected. No detectable necro-inflammatory foci or fibrosis were noted (Figs. 7 E and F).

The H&E splenic sections examination of infected non treated control subgroup (Ia) showed atrophic white pulp with diffuse congested red pulp infiltrated with lymphocytes.
with numerous reactive multinucleated giant cells. These megakaryocytes appeared heavily loaded with tachyzoites and surrounded by inflammatory cells with excess apoptosis and necrotic foci (Figs. 8 A and B). Meanwhile treated subgroups showed varying grades of histopathological improvement. In Septrin-treated subgroup (IIb), a greater number of lymphoid follicles in a relatively less atrophic white pulp was seen. Mild congested red pulp with some multinucleated large megakaryocytes engulfing few tachyzoites was noted, associated with moderated apoptosis and necrosis (Figs. 8 C and D). Correspondingly, in neem oil treated subgroup (IIC), the preserved white pulp was associated with hyperplastic lymphoid follicles. These follicles had prominent germinal centres. Mildly congested red pulp with scattered multinucleated giant cells with the scanty organism tachyzoites was evident. There was mild apoptosis and necrosis (Figs. 8 E and F), parenchymal architecture. Scanty small pseudocysts with few tachyzoites were detected. No detectable necro-inflammatory foci or fibrosis were noted (Figs. 7 E and F).

**DISCUSSION**

Up to the present, treatment of acute toxoplasmosis depends mainly on the synergistic effects of pyrimethamine and sulfadiazine or triple sulphonamides. Their severe side effects necessitate development of a safe and effective therapeutic agent to successfully eliminate toxoplasmosis[25]. In the current study, neem oil showed promising results as a potential natural anti-Toxoplasma agent. It resulted in reduction of the parasite load with amelioration of the histopathological changes, and prolongation of the mice survival without side effects.

Neem oil is known for its strong antioxidant and anti-inflammatory properties since the phytochemical analysis proved that it contains many bioactive components[25-27]. The effective use of neem extract is attributed to its active components that include free antioxidant, anti-inflammatory and anti-angiogenic actions with ability to modulate the immune system via various pathways[20,29]. It is worth mentioning that antimicrobial activity of essential oil is not assigned to a specific component but rather due to different components that target many functions in the cell[25,29].

In the current study, the low survival rate in mice with acute toxoplasmosis is explained by high invasion rate of host cell, uncontrolled parasite replication and tachyzoite dissemination to different organs[30]. So, prolonged survival may be attributed to the significant reduction in both tachyzoite count and viability observed in the current study that reached more than 75%. This reduction rate was comparable to that obtained using Cuminum cyminum seed oil in treatment of acute toxoplasmosis[30].

Our ultrastructural results may be explained by the fact that essential oils are typical lipophiles. They can interact and pass through the cytoplasmic membrane...
disrupting fatty acids, and phospholipids forming the cell membrane. Additionally, they can interact with different proteins embedded in the cell membrane increasing its permeability\textsuperscript{[11,12]}. The observed ultrastructural changes are comparable to those inflicted by essential oil of Pelargonium X. asperum on Toxoplasma tachyzoites in vitro\textsuperscript{[33]}. In our study, IFN-γ showed higher expression in mice treated with neem oil than in those treated with Septrin. Mahmoudvand et al.\textsuperscript{[34]} obtained similar results on using Zataria multiflora essential oil against acute toxoplasmosis. This rise could be one of the protective mechanisms induced by neem oil against toxoplasmosis that caused significant reduction in the parasite load and the ultrastructural changes observed in the current study. As one of Th1 cytokine, IFN-γ limits the parasite replication through activation of macrophages causing killing of the intracellular parasite\textsuperscript{[35]}. It was observed that IFN-γ also induced different effectors such as IFN-inducible GTPases which play a vital role in Toxoplasma growth suppression and facilitate its killing by immune cell\textsuperscript{[36]}. Neem extract immune stimulatory effect is explained by its content of limonoid epoxyazadiradione compound which causes inhibition of macrophages migration inhibitory factor resulting in release of proinflammatory cytokines including IFN-γ\textsuperscript{[37]}. Similar histopathological amelioration was observed by Gomaa et al.\textsuperscript{[38]} who used Cuminum cyminum seed oil as a therapeutic agent against toxoplasmosis\textsuperscript{[39]}. The observed anti-inflammatory effect of neem oil is explained by its high content of flavonoids, nimbins, and saponins. These components act as inhibitors of prostaglandin biosynthesis, and endoperoxides that are involved in inflammatory response\textsuperscript{[40]}. Furthermore, nimbin has the ability to supress the function of macrophages and neutrophils which play a vital role in inflammation\textsuperscript{[41]}. It is worth mentioning that nimbin is known for its antioxidant effects, by mitigating the production of reactive oxygen species leading to less tissue damage\textsuperscript{[42,43]}. Besides, Neem proved to enhance the natural defence by increasing production of GSH protecting the cell from harmful oxidative stress\textsuperscript{[44]}. From the compiled data of our study, we conclude that neem oil is an effective, safe anti-Toxoplasma agent due to its anti-inflammatory and antioxidant properties with minimal toxicity and no major side effects. 

**Author contributions:** Rashed HA contributed with Elmorsy EM in concept design, experimental study, data acquisition and analysis. Younis SS contributed in the experimental study. Abdel Bary A performed the histopathological examination. Elmorsy EM drafted the manuscript. All authors accepted the final version of the manuscript before publication.

**Conflict of interest:** Authors declare that there is no conflict of interest.

**Funding statement:** Authors received no specific funding.

**REFERENCES**

12. Shori AB, Baba AS. Antioxidant activity and inhibition of key enzymes linked to type-2 diabetes and...


