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RESEARCH ARTICLE

Myristica fragrans and Chronic Toxoplasmosis; *In Vivo* Parasitological, Histopathological and SEM Study

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Abstract

Toxoplasma gondii (*T.gondii*) is one of the most important protozoa causing abortion in pregnant women and fatal manifestations in immunocompromized individuals. The present study was conducted to assess the effect of *Myristica fragrans* (*M. fragrans*) alone and loaded on Chitosan nano particles (CS NPs) on chronic toxoplasmosis as a more effective safe candidate in comparison with spiramycin. One hundred and tenlaboratory bred Swiss albino mice were infected by oral inoculation of *T.gondii* tissue cysts, treated for 14 days starting from the 4th week post infection (p.i) and scarified at the 10th week p.i. The effect of drugs was assessed by counting the number of tissue cysts in homogenized brain tissues, histopathological examination and SEM of brain tissue. There was a significant decrease in the number of tissue cysts detected in the brain between the control group and all treated groups ($P<0.001$). Histopathological examination revealed marked improvement of inflammation in all treated groups and the SEM of brain tissues revealed remarkable changes in the shape and structure of the *T.gondii* tissue cysts.

Keywords *T. gondii – M. fragrans – CS NPs – in vivo – Me49 strain – Histopathological examination-*

Introduction

*T.gondii*is regarded one of the most important parasites causing abortion in pregnant women and fatal manifestations in immunocompromized individuals [1]. The main drug used in treatment of toxoplasmosis is spiramycin which is not effective and *T. gondii* had developed resistance against it. Another main problemis that the drug has no effect against the encysted stage of the parasite [2].

Therefore, the need for finding a new effective drug against *T.gondii* of natural source with little side effects becomes mandatory [3]. Nutmeg or *M.fragrans*was tested against *T.gondii in vitro*with promising results [4]. Chitosan is a biopolymer derived from chitin deacetylation which has wide anti-bacterial activity but lower toxicity toward mammalian cells [5].

Materials and Methods

Experimental Animals

One hundred and ten laboratory bred male Swiss albino mice were used**.** Each mouse was about 20-25 gm in weight. The animals were kept on a standard diet containing 24% protein, 4% fat, 4-5% fiber and water ad-libitumin in the biological unit of Theodor Bilharz Research Institute (TBRI). Animals were kept in suitable temperature of 24C°.

Ethical Considerations

The protocol of this study was approved by the ethical committee of Kasralainy school of

Medicine, TBRI and the Institutional Animal Care & Use Committee (IACUC) of Cairo University.

All the experiments were carried out according to The Clinical and Laboratory Standards Institute (CLSI) guidelines.

Drugs and Plant Extracts Preparation and Dose Adjustment

M.fragrans was purchased from a local market in Giza, Egypt and stored in Medicinal Chemistry Department, TBRI.

Preparation of the Extract

The plant material was dried in the shade, and ground in a grinder with a 2 mm diameter mesh, submitted for 3 h to water-distillation using a Clevenger apparatus to isolate the essential oil. The obtained oil was dried over anhydrous sodium sulphate and, after filtration, stored at +4 C until tested and analyzed. For preparation of the methanolic extract; 500 g of plant material were successively extracted with 1 l of methanol 85% by using a Soxhlet extractor for 72 h at a temperature not exceeding the boiling point of the solvent [6].

This step was repeated 3 times. Then the methanolic extract was filtered using Whatman filter paper (No: 1), then concentrated in vacuum at 40 C using a Rotatory Buchi Evaporator to dry. The residue obtained was lyophilized in a Modulyo freeze-dryer and the resulting powdered material was stored at 80 C until used [7].

Loading on Chitosan nanoparticles

Pure methanolic extract of *M.fragrans*was loaded on chitosan nanoparticles (CS NPs) in Nanotech Egypt Company. Aqueous triphenyl phosphate (TPP) solution (1.5 mg/ml) was added to chitosan solution (2 mg/ml, dissolved in 1% acetic solution) at volume ratio of 2:5 under magnetic stirring. Incorporation of TPP solution into chitosan solution containing 0.4 mg/ml *M.fragrans* at the same ratio was done.

The pH values of the nanoparticles suspension were adjusted to about 6.0 with NaOH, and oxidized *M. fragrans* solution (0.5, 1 and 2 mg/ml, dissolved in distilled water) was added dropwise into the nanoparticles suspension at oxidized *M. fragrans* / Chitosan ratios of 0.07:1, 0.14:1 and 0.28:1 (w/w) under magnetic stirring for 6 h, respectively. The mixture was then dispersed by ultrasonication for 60 s. Then the final volume of the mixture in each preparation was limited to 20 ml in order to yield uniform *M.fragrans* CLNP concentration [8].

Drug Preparation and Route

The pure methanolic extract of *M.fragrans* was put in shaker for 30 minutes to 1 hour to liquefy, and then the used amounts of the extract were dissolved in PBS. The extract was given orally to mice using eosophageal tube.*M.fragrans* CLNP was present in a liquid form. It was given orally to mice using eosophageal tube.

Dose Adjustment

M.fragrans was given at 2 different doses 250 mg/kg body weight/mouse and 500 mg/kg body weight/mouse [9, 10].*M.fragrans* CLNP was given at 2 different doses 250 mg/kg body weight/mouse and 500 mg/kg body weight/mouse.

Spiramycin

Spiramycin 3 M.I.U was used in a tablet form. It was provided by (Pharaonia Pharmaceuticals-analytical standard code: J01FA02). It was given at a dose of 50 mg/kg body weight/mouse.

Study design

T.gondii ME49 chronic strain was obtained from The National Research Institute in Guiza Egypt. Brains of 6-8 weeks infected mice with *T.gondii* tissue cysts were isolated after scarification & homogenization. Mice were infected by oral inoculation through eosophageal tube. 200 µl of PBS containing 100 tissue cysts were given to each mouse. Drugs were given from the 4th week p.i and continued for 14 days [11].

Experimental design

One hundred andten male albino mice were included in this experiment**;** they were divided into 11 groups 10 mice each as following:

Group I (Control group):

Ia: Infection control group; received no treatment.

Ib: Spiramycin control group; received spiramycin at a dose of 50 mg/kg body weight/mouse

Ic: Non-infected non-treated group (naïve mice).

Group II (Infected treated group):

II a: *M. fragrans* at a dose of 250 mg/kg body weight/mouse.

II b: *M. fragrans* CLNP at a dose of 250 mg/kg body weight/mouse.

II c: *M. fragrans* at a dose of 500 mg/kg body weight/mouse.

II d: *M. fragrans* CLNP at a dose of 500 mg/kg body weight/mouse.

II e: *M. fragrans* at a dose of 250 mg/kg body weight/mouse in combination with spiramycin at a dose of 50 mg/kg body weight/mouse.

II f: *M. fragrans* CLNP at a dose of 250 mg/kg body weight/mouse in combination with spiramycin at a dose of 50 mg/kg body weight/mouse.

II g: *M. fragrans* at a dose of 500 mg/kg body weight/mouse in combination with spiramycin at a dose of 50 mg/kg body weight/mouse.

II h: *M. fragrans* CLNP at a dose of 500 mg/kg body weight/mouse in combination with spiramycin at a dose of 50 mg/kg body weight/mouse.

Parasitological Evaluation

The number of tissue cysts was counted in the homogenized brains to evaluate the reduction in their numbers per gram tissue.

Histopathological Examination

Histopathological examination of brain tissues using standard light microscope was done to evaluate the degree of inflammation in different groups. Brain inflammation was assessed for each study group [12].

Electron Microscopy

Scanning electron microscopy was performed to detect morphological changes that occurred to tissue cysts in response to drugs [13].

Statistical analysis of data

The mean and the standard deviation (±SD) for the parametric numerical data were calculated. ANOVA test was used to assess the statistical significance between all experimental groups, followed by Post Hoc tests to compare significance between all groups. P-value was considered significant at < 0.05. The histopathological results were analyzed using one of the non-parametric tests (Kruskal- Wallis test) and the results were expressed in median.

Results

Parasitological Results

Quantitative assessment of the number of *T.gondii* tissue cysts in brain sections of infected mice after administration of drugs at different daysp.i was performed to count the number and to calculate the percentage of reduction. Data was represented in Table 1.

Table 3: Number of T. gondii tissue cysts/20µl brain homogenate in groups treated with M. fragrans (p<0.001 significant decrease than IC; ap<0.05 significant decrease than S; bp<0.01 significant increase than S).**

	N	Minimum	Maximum	Mean± SEM	% reduction	F-value	*p-value*
IC	10	1045.00	4550.00	2729.50±426.10	1205.19850	6.925	<0.001
S	10	1156	3125	1818.00±329.04**	33.4%		
M2	10	720	1550	1120.00±110.60^{**,a}	58.9%		
MN2	10	576	1074	870.00±79.57^{**,b}	61.4%		
M5	10	512	1600	1053.33±192.01^{**,a}	61.4%		
MN5	10	378	1600	934.67±174.44^{**,b}	65.7%		
S+M2	10	528	1560	1119.00±190.58^{**,a}	59%		
S+MN2	10	414	1492	1018.86±164.42^{**,a}	62.7%		
S+M5	10	420	1545	932.50±189.95^{**,b}	65.8%		
S+MN5	10	420	1240	815.00±139.88^{**,b}	70.1%		

Histopathological results

Histopathological examination of brain tissue sections was done for all groups. Evaluation depended on the intensity of inflammation, the activity of inflammation and the heaviness of the infection. The degree of

inflammation was classified into grades 1, 2 and 3 according to heaviness of the cellular infiltration and presence of lymphocytic aggregation. The activity of inflammation was classified into grades 1, 2 and 3 according to presence of neutrophils [12]. The heaviness of the infection was classified into grades 1, 2 and 3 according to the number of tissue cysts Fig.1.

SEM results

Brain samples from the IC group, the group treated with spiramycin and the group treated with *M.fragrans* CLNP at a dose of 500 mg/kg in combination with spiramycin; were subjected to examination under SEM. Fig.1.

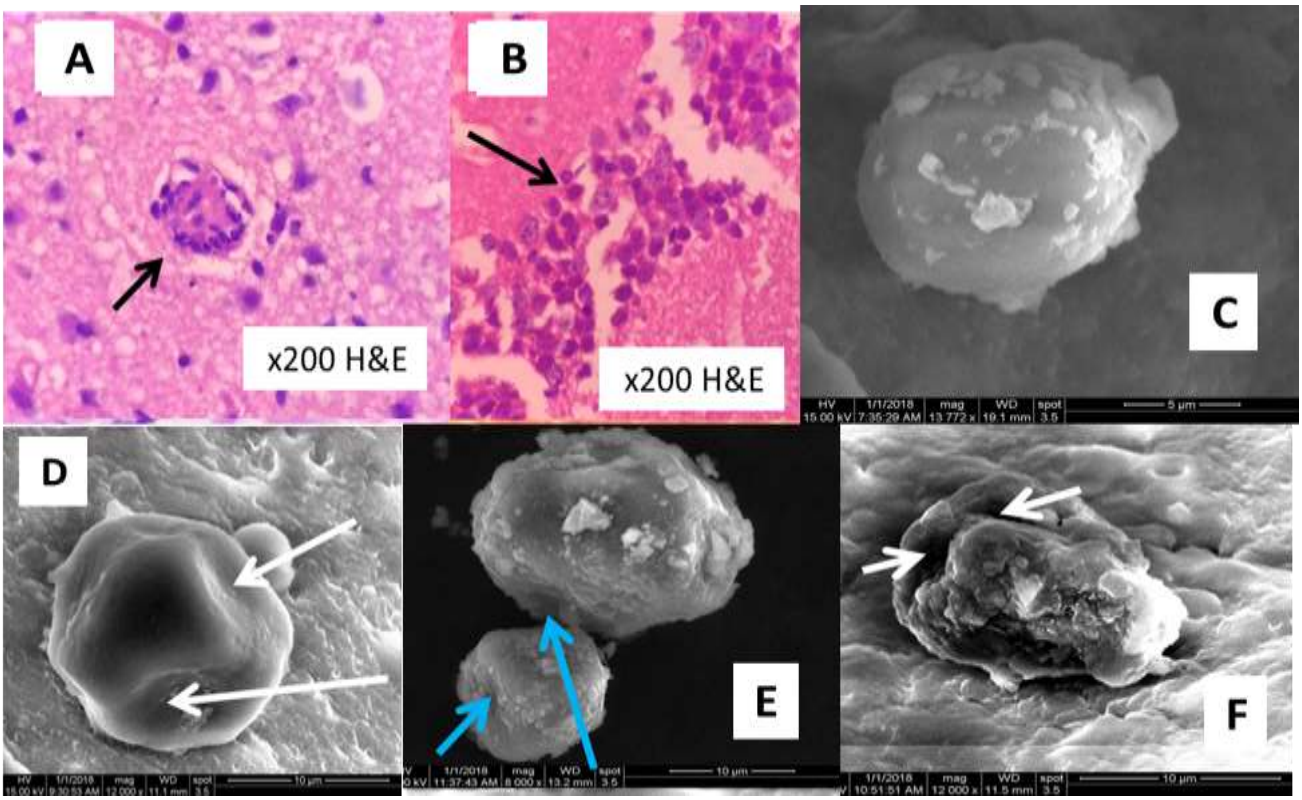

Fig. 1: A: Histopathological examination showingperivascular cellular infiltration (arrow) seen in the group treated with *M.fragrans*. B: Histopathological examination showingmoderate inflammation seen in the group treated with *M.fragrans*. C: SEM picture of tissue cyst of *T.gondii* from the IC group appeared intact, rounded and surrounded with intact cyst wall with smooth surface.D: SEM picture of tissue cysts of *T.gondii* from the group treated with spiramycin appeared humiliated with large holes and dimples (White arrows).E & F: SEM picture of tissue cysts of *T.gondii* from the group treated with *M.fragrans* in combination with spiramycin. Cysts appear distorted (White arrows) with dimples (Blue arrows) and projections.

Discussion

All groups treated with *M.fragrans* showed a significant reduction of the number of *T.gondii* tissue cysts in brain with percentage reduction ranging from **58.9%** to **70.1%.** They also showed a statistically significant reduction when compared to **33.4%** detected in the spiramycin treated group. This reduction in the number of tissue cysts might be due to the active ingredients of *M.fragrans* methanolic extracted including: limonene, eugenol and terpinen-4-ol. Limonene modulates immune responses, increases primary and secondary antibody responses and enhances macrophage phagocytosis and microbicidal activity [14].

Eugenol affects energy generation by cells by inhibition of glucose uptake or utilization of glucose as well as affection of the membrane permeability [15]. Terpenin-4-ol showed the blockage of mitochondrial biofilm respiration and inhibition of enzyme activity [16].Histopathological examination of brain tissue sections showed that *M.fragrans* alone showed slight reduction of inflammation, however, *M.fragrans* loaded on CS NPs or combined with spiramycin showed marked resolution in brain inflammation.

This interestingly marked effect of *M.fragrans*methanolic extract on the degree of inflammation might be due to the effect of its myristicin component; which has anti-inflammatory properties related with its inhibition of NO, cytokines, chemokines, and growth factors in dsRNA-stimulated macrophages via the calcium pathway [17].

In contrary to our results, *M.fragrans* was tested against *T.gondiiin vivo* and showed minimal effectiveness; however, they used ethanolic extract [18].*In vitro,* nutmeg oil (*M.fragrans*) extract was tested against *T.gondii*tachyzoites and reported similar results. Nutmeg oil showed significant difference of inhibition against the *T.gondii* ($p<0.01$) in a dose dependent manner [4].

Also; *M.fragrans* was tested against anisakis L3 larvae and induced high death rate [19].Apart from parasitological field; *M.fragrans* showed strong antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae* and multi-drug resistant *Salmonella typhi* and *Helicobacter pylori* [20]. Nutmeg essential oil had been shown to affect the growth and survival of *Yersinia enterocolitica* & *Listeria monocytogenes* [21].

It revealed a significant effect on inhibiting the growth of *Escherichia coli* and *Staphyloccocus aureus* [22]. *M. fragrans* showed antifungal activity against *Colletotrichumgloeosporoides*, *Colletotrichummusae*, *Fusarium oxysporum*, *Fusarium semitectum*, *Aspergillus niger*, &*Aspergillus glaucus* [23]. Also, mace methanol extract showed a significant antifungal activity against *Candida albicans* & *A.niger* [24].

No difference was found in our results between groups treated with *M.fragrans* alone and the groups treated with the extract loaded on CTNP; and this is attributed to several limitations, such as low solubility at neutral pH, low surface area and low porosity [25].However when CS NPs loaded on spiramycin and tested against chronic *T. gondii* infection; it significantly decreased the mortality rate of mice infected with Me49 strain compared to high mortality rate of mice in the infected control subgroup [26].

Our results showed that spiramycin had a very little effect on chronic toxoplasmosis (**33%** percentage reduction of the number of tissue cysts); and this is because spiramycin is unable to reach effective concentrations in the brain due to the presence of the efflux transporters multidrug-resistant protein 2 and P-glycoprotein, however; spiramycin uptake in the brain can be augmented by co-administration of a drug that inactivate the efflux pump as metronidazole [27].

Conclusion

The present work was done to assess the effect of *M.fragrans* alone and loaded on CS NPs in chronic toxoplasmosis in comparison to spiramycin. All groups treated with *M. fragrans* showed a significant reduction of the number of *T. gondii* tissue cysts in brain with percentage reduction ranging from **58.9%** (in the group treated with the dose 250 mg/kg body weight/mouse) to **70.1%** (in the group treated with *M. fragrans* CLNP 500 mg/kg body weight/mouse and spiramycin).

Histopathologically; *M .fragrans* alone showed slight reduction of inflammation; however, when *M. fragrans* was loaded on CS NPs or combined with spiramycin it showed marked resolution in brain inflammation. As regards SEM examination of *T. gondii* tissue cysts obtained from brain tissues of the group treated with the combination between *M. fragrans* CLNP at a dose of 500 mg/kg and spiramycin, it showed distorted architecture. Their surfaces show dimples in addition to arising of small projections.

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All manuscript authors contributed to every activity of it; idea of paper, study design, collection of materials, methodology, writing the paper and revising it. The authors declare that they have no competing interests.

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