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# *In-vivo* Comparison of the Efficacy of *Nigella sativa*, Thymoquinone and Capsaicin against *Toxoplasma gondii* with Pyrimethamine-Sulfadiazine

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**ABSTRACT**

Toxoplasma gondii (T. gondii) is estimated to infect a third of the world's population although the majority of the infections are largely asymptomatic. The combination of pyrimethamine and sulfadiazine is considered the treatment of choice. These drugs cannot eliminate dormant tissue cysts. The aim of this study is to evaluate the therapeutic effects of Nigella sativa oil, thymoquinone and capsaicin. The study was carried out as two separate experiments. Balb/c mice were injected intraperitoneally with $10^5$ and $10^3$ T. gondii. After 24 hours, treatment was started with different doses of Nigella sativa oil, thymoquinone and capsaicin for 10 days. The effects of these components were evaluated compared to the combination of PYR-SDZ. Mortality and tachyzoite amounts of the mice were calculated for 30 days. In both experiments, the mice in the PYR-SDZ group lived to the fullest. Then the mice in the capsaicin group lived the longest. Since early losses occurred in both experimental groups, tachyzoite count was not performed in groups other than PYR-SDZ. Tachyzoites were not observed in the PYR-SDZ groups after day 10. It was determined that after the PYR-SDZ treatment of toxoplasmosis, capsaicin displayed the highest probability of survival. In conclusion, the combined use of capsaicin and PYR-SDZ can increase life expectancy by reducing the side effects of the drug.

**Keywords:** Capsaicin, Nigella sativa, Pyrimethamine-Sulfadiazine, Thymoquinone, Toxoplasma gondii.

## Introduction

Toxoplasmosis, caused by *Toxoplasma gondii*, is a life-threatening disease that affects healthy and immunocompromised people in many countries (1). The great majority of human *T. gondii* infection occurs either by ingestion of oocysts that are generated in the felid intestine and spread throughout the environment via feces or ingestion of *T. gondii* tissue cysts in undercooked meat. Congenital infection occurs through vertical transmission when a previously uninfected mother is infected during pregnancy. Although it is not very common, it can also be transmitted by blood transfusion and organ transplant (2). Most cases of toxoplasmosis in healthy individuals are asymptomatic. However, congenital toxoplasmosis may include hydrocephalus, microcephaly, intracranial calcifications, retinochoroiditis, strabismus, blindness, epilepsy, psychomotor and mental retardation (3). In most hosts, *T. gondii* produces a lifelong latent infection of tissues such as skeletal muscle, heart muscle, or the central nervous system, which includes the brain, spinal cord, and retina (4). Tissue cysts or oocysts invade the host cells after ingestion and differentiate into rapidly dividing tachyzoites within the host cells. With the host's immune response to this pathogen, clinical signs of infection occur. The tachyzoites differentiate into bradyzoite forms hidden within the parasitophore vacuole. This differentiation can be enhanced by exposure of the organism to stress conditions, such as an immune response to tachyzoites. Tissue cysts can remain indefinitely throughout the life of the host. If a person is immunocompromised, these tissue cysts act as a reservoir from which disseminated or local infections can develop. (5).

The drugs recommended for classical toxoplasmosis chemotherapy are pyrimethamine and sulfadiazine. Drugs commonly used in the treatment of toxoplasmosis inhibit the proliferation of the parasite and thus protect organs from damage, but cannot completely eliminate it from the host organism. Also, PYR can lead to bone marrow suppression and hematological toxicity (6). Quite a large number of

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plants have been identified and studied, which produce natural products with significant anti-parasitic action. Extracts of plants such as *Artemisia annua*, *Zingiber officinale*, *Sophora flavescens*, Aiton, *Eurycoma longifolia*, *Nigella sativa*, *Bunium persicum* have been used both in vitro and in vivo for the treatment of toxoplasmosis. (3,7). Progress in toxoplasmosis vaccine development has been ongoing for decades, but an effective vaccine for clinical use is still lacking. Research and development of a new safe and effective drug with low toxicity seems urgent and vital. In addition, medicinal plants will be used for the development of future studies and new treatment strategies for people susceptible to T. gondii (3).

*Nigella sativa* oil is an enhancer of natural killer (NK) cells with potential applicability in immune therapy; its effect has been shown to increase the rate of interferon-gamma (IFN-y), helper T cell (T4) and suppressor T cell (T8) in humans (8). Many pharmacological effects of thymoquinone (TQ), such as antioxidant, anti-inflammatory, immunomodulatory, antihistamine, antimicrobial and anti-tumor effects, have been investigated and its protective effects on many organs have been proven (9,10). Many of the biological effects of capsaicin (CAP) have been extensively studied. It has pharmacological effects such as analgesic, anti-inflammatory, anticarcinogenic, antilipemic, antioxidant, anti-dyspeptic, antibacterial, and immunosuppressive (11-13).

In this study; considering the need for alternative drugs with less side effects against
*T. gondii*, we aimed to compare the efficacy of NSO, TQ, CAP compounds and PYR-SDZ drug combination was compared in acute toxoplasmosis *in vivo*.

## Materials and Methods

***T. gondii* Strain:** Standard Turkish TR01 strain tachyzoites obtained from parasitology strains of national type culture collection of National Reference Parasitology Laboratory of Türkiye Public Health institution were used. TR01 strain was maintained by inoculated intraperitoneally to Balb/c mice every 3-4days.

**Experimental Design:** This study was conducted in the experimental animal unit after the approval of Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee with the date 03.12.2020 and numbered 2020/11-16. Provided from Experimental Animals Application and Research Center, 18-22 g. weight and 6-8 weeks old female Balb/c mice were used. Mice were housed in euro Standard type 3 transparent poly carbonate cages in 12 hours of light and 12 hours of darkness and at 18-24ºC in accordance with the conditions of the research laboratory, supplied with Standard pellet food and water.

The study consisted of, two separate experimental groups were formed. In experiment 1, a total of 64 female Balb/c mice were infected with $10^5$ tachyzoites. Before the mice were infected with tachyzoites, the injection site was cleaned with ethanol for possible contamination and infected intraperitoneally. The mice were divided into eight groups (n=8); PYR-SDZ (12.5-200 mg/kg/day) (Santa Cruz Biotechnology, U.S.A.); NSO (1000-500 mg/kg/day) (Origo, Turkey); TQ (100-50 mg/kg/day) (Santa Cruz Biotechnology, U.S.A.); CAP (50-25 mg/kg/day) (Santa Cruz Biotechnology, U.S.A.); and untreated control group. In experiment 2, a total of 20 female Balb/c mice were diluted with saline in sufficient amount according to the number of tachyzoites and drawn into a one cc insulin syringe with $10^3$ tachyzoites in 100µl. Before the mice were infected with tachyzoites, the injection site was cleaned with ethanol for possible contamination and infected intraperitoneally. The mice were divided into four groups: PYR-SDZ (12.5-200 mg/kg/day); NSO (1000 mg/kg/day); TQ (100 mg/kg/day); CAP (50 mg/kg/day).

Drug doses were calculated according to the weight of the mice and prepared daily. PYR-SDZ with dimethyl sulfoxide; NSO with olive oil; TQ and CAP were also diluted with saline at 45 ℃. A homogeneous mixture was obtained by vortexing these solutions for five minutes. Treatment was started after 24 hours in both experimental groups. The drugs were administered as a single dose, in a final volume of 0.2 ml and at the same time each day for 10 days orally using gavage (F.S.T, Barrel tip). Infected mice were observed to survive for 30 days. In both experiments, one mouse was randomly selected on certain days of treatment (in the first experiment, on the 5th, 10th, 15th, 20th, 25th and 30th days; in the second experiment, on the 15th, 20th, 25th and 30th days). They were anesthetized intraperitoneally with a cocktail of ketamine (Ketalar®, Parke Davis and Co. Inc., 50mg/kg) and xylazine (Rompun®, Bayer 5 mg/kg). One ml of saline was injected into the peritoneum. The fluid taken from the exudates was examined for the presence of tachyzoites in the Thoma slide.

**Statistical Analysis:** For the statistical analyses, SPSS software version 15.0 was used. The 95% confidence interval and $p<0.05$ significance level were taken into account. Survival probabilities were calculated using Kaplan-Meirer survival

analysis. Probability of survival between groups was compared with the Log Rank test.

## Results

The longest life span was observed in the PYR-SDZ group with 30 days, then the CAP50 and TQ100 groups with 5,625 days, and the shortest was NS1000 group with 4 days (Fig. 1, Table1). In the PYR-SDZ group, the first mouse died on the 25th day and the other seven mice survived to the end of the experiment. Tachyzoite counting was performed only in the PYR-SDZ group, because of early dead in other groups. On the 5th day of the treatment, approximately 3-5 tachyzoites were counted in each medium-sized square of the Thoma slide in the exudate of the mouse whose peritoneum was opened. Necessary calculations have been made. $1x10^3$ (800-1300) tachyzoites were counted in an average of 1 $mm^3$ of peritoneal exudate. After the 10th day, tachyzoites were not observed in the peritoneal exudates of the mice in the PYR-SDZ group.

According to the mean probability of survival, the PYR-SDZ and NS1000 groups were found to be significant with the other groups. ($p<0.001$). Compared with the control group, the most significant PYR-SDZ ($p<0.001$) was followed by KAP50 ($p<0.003$), KAP25 ($p<0.006$), and TQ100 ($p<0.006$), respectively (Table 2).

In the experiment 2, the longest PYR-SDZ (30 days), followed by CAP50 (7,125 days), and the shortest control group (5 days) survived (Fig. 2, Table 3). Tachyzoite counting was performed only in the PYR-SDZ group. Because early losses were seen in other groups. On the 15th day of treatment, the peritoneum of a randomly selected mouse was opened and no tachyzoites were found in the peritoneal exudate. In terms of probability of survival, the control group and all other groups were significant ($p<0.05$) (Table 4).

## Discussion

Therapeutic studies have been carried out on strains of *T. gondii* using many natural plant extracts. *T. gondii* has become resistant due to long-term drug use. In addition, high costs and less access to chemical drugs increase the interest in the healing effects of medicinal plants, especially in underdeveloped and developing countries. Pharmaceutical industries are also working on alternative solutions against parasitic diseases with low side effects and low cost. In recent years, a lot of research has been done to detect the anti-parasitic properties of plants. (3,7). So far plants such as *Artemisia annua*, *Zingiber ofcinale*, *Sophora flavescens*, *Aiton* and *Eurycoma longifolia*, Myrrh, *Piper nigrum*, *Capsicum frutescens*, *Curcuma longa*, *Azadirachta indica* and *Melia azedarach* and *Nigella sativa* have been studied and shown to be effective against *T. gondii* (3,14).

Drugs recommended for the treatment or prophylaxis of toxoplasmosis are PYR-SDZ, trimethoprim-sulfamethoxazole (TMP-SMX) combinations as well as spiramycin. Second-line therapy drugs consist of atovaquone, epiroprim, azithromycin, and clarithromycin. Although all these drugs can effectively cure toxoplasmosis, approximately 40% of patients discontinue treatment due to serious toxic side effects, and up to 80% may relapse without long-term treatment. However, current standard therapies against toxoplasmosis are limited, and drugs have severe side effects and low efficacies. (15-16). Combined treatments were used to reduce the side effects of PYR-SDZ, which is often preferred for the treatment.

Koksal et al., investigated the survival of mice infected with *T.gondii* with PYR-SDZ, and levamisole and *Echinacea*. Compared to PYR-SDZ, the combination of PYR-SDZ+levamisole was found to be more successful, increasing the lifespan of mice from 33.3% to 89.9% (17). Similarly, in infected mice treated with PYR-SDZ+fluconazole and PYR+fluconazole, the combinations proved highly effective against *T. gondii* (18). Propranolol has shown prophylactic and therapeutic effects together with PYR against *T. gondii*. (15). Eissa et al. (2015) investigated the efficacy of miltefosine and SDZ in acute and chronic experimental toxoplasmosis. Miltefosine do not verify anti-parasitic activity in acute toxoplasmosis, but its effectiveness against the cyst stage of the parasite in chronic infection has been proven (19).

The effectiveness of many plant extracts against *T. gondii* has been evaluated in numerous in vivo studies to date. However, there is no study on the effectiveness of TQ, which is the main bioactive component of NSO, and CAP, which is the active ingredient of hot pepper against *T. gondii*.

NS seed has been used for centuries in different civilizations around the world to treat a variety of animal and human disease. NS has anti-inflammatory, antimicrobial and disinfectant effects. So far, its effect on neurological and mental diseases, cardiovascular disorders, cancer, diabetes and

**Table 1.** Experiment 1, Survival Time in $10^5$ Tachyzoite Infected Groups By Time

| | Estimate | Std. Error | 95% Confidence interval | |
|---|---|---|---|---|
| | | | Lower Bound | Upper bound |
| PYR-SZD | 30,000 | 0,000 | 30,000 | 30,000 |
| NSO1000 | 4,000 | 0,000 | 4,000 | 4,000 |
| NSO500 | 4,875 | 0,125 | 4,630 | 5,120 |
| TQ100 | 5.625 | 0,189 | 5,130 | 5,870 |
| TQ50 | 5,250 | 0,164 | 4,929 | 5,571 |
| CAP50 | 5,625 | 0,189 | 5,130 | 5,870 |
| CAP25 | 5,500 | 0,183 | 5,266 | 5,984 |
| CONTROL | 4,500 | 0,189 | 4,130 | 4,870 |
| OVERALL | 8,156 | 1,045 | 6,109 | 10,204 |

**Table 2.** Experiment 1, Pairwise Comparisons of $10^5$ Tachyzoite Infected Groups

| Pairwise Comparisons | | | | | | | |
|---|---|---|---|---|---|---|---|
| | NS1000 | NS500 | TQ100 | TQ50 | CAP50 | CAP25 | CONTROL |
| PYR-SZD | <0,001 | <0,001 | <0,001 | <0,001 | <0,001 | <0,001 | <0,001 |
| NS1000 | | 0,001 | <0,001 | <0,001 | <0,001 | <0,001 | 0,025 |
| NS500 | 0,001 | | 0,020 | 0,089 | 0,020 | 0,008 | 0,117 |
| TQ100 | <0,001 | 0,020 | | 0,317 | 1,000 | 0,626 | 0,006 |
| TQ50 | <0,001 | 0,089 | 0,317 | | 0,317 | 0,143 | 0,015 |
| CAP50 | <0,001 | 0,020 | 1,000 | 0,317 | | 0,626 | 0,003 |
| CAP25 | <0,001 | 0,008 | 0,626 | 0,143 | 0,626 | | 0,006 |
| CONTROL | 0,025 | 0,117 | 0,006 | 0,015 | 0,006 | 0,003 | |

**Table 3.** Experiment 2, Survival Time In $10^3$ Tachyzoite Infected Groups By Time

| | Estimate | Std. Error | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Lower Bound | Upper Bound |
| PYR-SZD | 30.000 | 0.000 | 30.000 | 30.000 |
| NS1000 | 6.750 | 0.265 | 6.230 | 7.270 |
| TQ100 | 7.000 | 0.000 | 7.000 | 7.000 |
| KAP50 | 7.125 | 0.513 | 6.119 | 8.131 |
| CONTROL | 5.000 | 0.000 | 5.000 | 5.000 |
| OVERALL | 12.809 | 2.755 | 7.409 | 18.208 |

inflammatory conditions, as well as on various infectious diseases due to bacteria, fungi, parasites and viral infections has been demonstrated. This plant also regulates the immune system, strengthens cellular immunity by increasing the production of interleukin (20). The effectiveness of NSO against the *T. gondii* Me49 (cyst- shaped) strain was proven, and effectively increased the survival time of mice with bradyzoites in the brain (21). It was also found that cellular immunity was stimulated with a significant increase in IFN-γ level (22). In addition, Ünal, (2012) used the combination of PYR-SDZ+NSO against *T. gondii* and the survival in mice increased from 33.3% to 66.6% (23). The efficacy of NSO has also been investigated in other parasitic agents. For example, NSO has been reported to reduce the number of adults and eggs deposited in both the liver and gut in mice infected with *Schistosoma mansoni.* (24). In another study, NSO reduced the percentage of fecal eggs in children naturally infected with cestode worms (25). Zaki and El Amir (2018) reported that cysteine protease inhibitor combined with NSO showed high

**Table 4.** Experiment 2, Pairwise Comparisons of $10^3$ Tachyzoite Infected Groups

| Pairwise Comparisons | | | | | |
|---|---|---|---|---|---|
| | PYR-SZD | NS1000 | TQ100 | CAP50 | CONTROL |
| NS1000 | 0,018 | | 0,317 | 0,544 | 0,014 |
| TQ100 | 0,014 | 0,317 | | 0,886 | 0,014 |
| CAP50 | 0,013 | 0,544 | 0,886 | | 0,014 |
| CONTROL | 0,014 | 0,014 | 0,014 | 0,014 | |

**Fig. 1.** Experiment 1, number of live mice in $10^5$ tachyzoite-infected groups by time

improvement in the treatment of cryptosporidiosis in mice (26). In our first experiment with NS, four days of survival was observed with 1000 mg, although the dose was halved, life expectancy was prolonged (4,875 days). Here, it was determined that the high dose had a negative effect on the life span, and a longer period was found even in the control group (4,5 days).

TQ, the most abundant component of essential oil, is the biologically active compound of NS seeds. TQ prevents weakening of the immune system. At the same time, it protects healthy cells from oxidative damage and toxic side effects, allowing cells to heal for a long time. (27). Although there is no study yet to determine the effectiveness of TQ against *T. gondii*, there are some experiments on other parasites. TQ was tested against *Entamoeba histolytica* and *Giardia lamblia* using in vitro susceptibility tests and parasite mortality was calculated. TQ was shown to have a stronger effect on *E .histolytica* (28). TQ was tested on *Fasciola gigantica* in vitro and all worms survived after 3 hours of exposure but a significant reduction in worm motility was observed in thymoquinone. A significant decrease in glutathione-S-transferase and superoxide dismutase activity and a decrease in glutathione (GSH) levels were observed. Also, inhibition of Cathepsin L gene expression was also evident in thymoquinone-treated worms (29). The anti-microsporidial effect of TQ against *Encephalitozoon intestinalis* was evaluated and it was observed that it decreased spore density especially at 30 μM

**Fig. 2.** Experiment 2, number of live mice in $10^3$ tachyzoite-infected groups by time

concentration. Although thymoquinone exhibits potent anti-microsporidial effects, high concentrations were also found to be toxic to host cells (30). While studies reported that thymoquinone is more effective than *N. sativa* extracts, some of them used only *N. sativa* instead of thymoquinone and their effectiveness was found to be very low. This is due to the fact that these studies are usually only done in vivo and show different effects on different parasites. We obtained better results with TQ than with NS in both experiments. Especially the results between the NS 1000 mg group and the TQ groups were found significant.

The CAP gives the peppers the characteristic pungent aroma. Its effects on the human body have been studied for over a century. It is an odorless, fat-soluble compound that is rapidly absorbed by the skin (31). Although it can cause skin irritation, it is useful in relieving different neuropathic pain conditions. It has been used in topical creams to treat chronic pain syndromes in the skin (32,33). The capsaicin content of twenty-nine varieties of *Capsicum* pepper extract was measured and its bactericidal and antifungal effects were determined at different concentrations (34). The effects of CAP on *T. gondii* were not found in our literature review. There is only some information about the effect on trypanosomatid parasites. A moderate antiparasitic effect against *Leishmania infantum* was obtained in combination with capsaicin meglumine antimonate. CAP was used in the oral treatment of *Trypanosoma cruzi* and found

effective, and is 57 times more potent than benznidazole currently used to treat Chagas disease (35). CAP may be useful in pain management at low concentrations. However, it has adverse effects at high concentrations. Oral LD50 values for CAP in mice are118.8 mg/kg, and prolonged administration of high doses can cause chronic gastritis, neurotoxic effects, and kidney and liver damage (36). In low doses, it has numerous benefits on the gut microbiota, especially targeting metabolic and inflammatory diseases (37). In our study, CAP was tried in two different doses (25 and 50 mg/kg/day) and life span was prolonged in both groups, while CAP50 mg doses was found to be significant compared to the control group (Table 2).

In our study, we investigated the effectiveness of herbal extracts at different doses. This is the first research to investigate the effectiveness of TQ and CAP against *T. gondii.* After PYR-SDZ, CAP50 exhibited the highest probability of survival. Also, the significant and known effect of PYR-SDZ on *T. gondii* was proven again with this study. It is thought that conducting studies in which PYR-SDZ and plant extracts are used together will be beneficial in terms of increasing life expectancy.

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