Anti-Toxoplasma Activity of Estragole and Thymol in Murine Models of Congenital and Noncongenital Toxoplasmosis

Authors: Claudio B. S. Oliveira, Ywlliane S. R. Meurer, Thales L. Medeiros, Adrian M. Pohlit, Murilo V. Silva, et. al.

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ABSTRACT: Toxoplasmosis is caused by Toxoplasma gondii, an obligate intracellular protozoan. Normally benign, T. gondii infections can cause devastating disease in immunocompromised patients and through congenital infection of newborn babies. Few prophylactic and therapeutic drugs are available to treat these infections. The goal of the present study was to assess the anti-Toxoplasma effects in a congenital and noncongenital model of toxoplasmosis (using ME49 strain), besides assessing immunological changes, in vitro cytotoxicity, and in vivo acute toxicity of these compounds.

Toxoplasma gondii is a widespread intracellular protozoan parasite capable of infecting virtually any nucleated cells of warm-blooded hosts including humans (Schultz et al., 2014). As an opportunistic human pathogen, T. gondii causes a devastating disease in immunocompromised individuals, especially HIV/AIDS patients and congenitally infected neonates (Montoya and Liesenfeld, 2004). In the United States, its prevalence has been estimated to be 15.8% among people of 12-49 yr of age (Jones et al., 2007). Most infected newborns have no symptoms at birth, but serious clinical manifestations can develop during childhood and early adulthood (Robert-Gangneux and Darde, 2012). Infection can occur congenitally or be acquired orally through contamination of food with oocysts released from cat feces and tissue cysts present in raw and undercooked meat. Finally, it can also be transmitted via the placenta, when acute maternal infection occurs during pregnancy (Dejérelli et al., 2003; Carruthers and Suzuki, 2007). The risk of transmission during pregnancy is mostly restricted to new infections. The parasite reaches the fetus via the placenta, causing varying degrees of damage (Sonda and Hehl, 2006). When maternal infection occurs in the first trimester of pregnancy, the occurrence of vertical transmission is less probable than in the third quarter, but the severity of the disease in newborns is greater (Dubey and Jones, 2008; Costa-Silva and Pereira-Chioccola, 2010).

Even with extensive research into the biology and physiology of T. gondii, antifolate combination therapy (e.g., pyrimethamine and sulfadiazine) is the first line of treatment and is poorly tolerated or causes several allergic reactions (Kaye, 2011). These limitations, combined with the fascinating properties of coccidian parasites (Cardoso et al., 2014), have accelerated interest in investigating the mechanisms governing the infection biology of these pathogens and making prospecting for new drug targets a critical goal (Kaye, 2011).

Among the species used in traditional medicine in the northeastern region of Brazil are Croton zehntneri Pax and K. Hoffm. and Lippia sidoides Cham., popularly known as “canelinha” and “alerce-pimenta”, respectively. These species have shown antibacterial action and against promastigotes and amastigotes of Leishmania amazonensis (Costa et al., 2008; Medeiros et al., 2011). The volatile oils (VOs) of these plants also exhibit antimalarial activity against Plasmodium berghei in mice and Plasmodium falciparum in vitro (Mota et al., 2012). These parasites belong to the phylum Apicomplexa, as do Toxoplasma spp. The main components of the VOs from Lippia sidoides and C. zehntneri are, respectively, thymol and estragole, which have in vitro antimalarial activity; however, anti-Toxoplasma activity has not been reported for these compounds. The aim of the present research is to investigate the anti-Toxoplasma activity in a congenital and noncongenital model of toxoplasmosis, using ME49 strain, besides assessing immunological changes, in vitro cytotoxicity, and in vivo acute toxicity of commercial estragole and thymol.

This work is important for providing new treatment options for a disease with limited options and recognized toxicity. These molecules may eventually be incorporated in the therapeutic regimen of toxoplasmosis in the general population and in pregnant women, which appear as important risk group because of the chance of miscarriages and birth defects.

MATERIALS AND METHODS

Phytochemicals

Estragole and thymol were provided by Kaapi (São Paulo State, Brazil). Their identity and >99.9% purity were ascertained as previously described (Mota et al., 2012).

Animals

Female C57BL/6 strain and outbred Swiss Webster mice (6–8 wk old and 20–25 g weight) were used for toxicity and antiprotozoal assays. Mice
were housed with drinking water and regular mouse feed ad libitum. The number of experimental mice for each procedure was calculated on the basis of previous studies (see below) (Oliveira et al., 2014). The animals were observed daily for mortality and morbidity. Surviving animals were euthanized for quantification of the parasite burden in brain tissues.

**Cytotoxicity assay**

For the cytotoxicity assay, the method used was adapted from a previously described procedure (Oliveira et al., 2014). Established cell lines selected for in vitro analysis were human hepatoma cells (HepG2; Sigma-Aldrich, St. Louis, Missouri), human cervical carcinoma cells (HeLa, Sigma-Aldrich), and peripheral murine macrophages obtained from mice (Mus musculus). Cells were seeded in 96-well flat-bottom tissue culture plates in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO Inc., Grand Island, New York) supplemented with 40 mg/L gentamicin and 10% fetal bovine serum (GIBCO Inc.). The cells were incubated in an atmosphere of 5% CO₂ at 37 °C and were subcultured every 7 days.

Estragole was dissolved (1.0%) in dimethyl sulfoxide (DMSO; Sigma-Aldrich, São Paulo, Brazil) and xylazine (Calmium, Uniação Química, São Paulo State, Brazil) intraperitoneally and then humanely killed by cervical dislocation. Surviving animals after 30 days were also anesthetized and then euthanized as described above.

**Anti-Toxoplasma gondii activity of the compounds in vivo**

ME49 strain of *T. gondii* was used. The mildly virulent ME49 strain of *T. gondii* (genotype II) was obtained from the brains of chronically infected Swiss Webster mice. Cells were quantified in brain suspensions, and after standard dilution, each mouse was infected by gavage. For treatments, 200 μl of thymol, estragole, or vehicle were dissolved as described below and administered by oral or subcutaneous routes.

The effects of each compound were explored in ME49 strain-infected mice by gavage and subcutaneous administration. Mice were infected (n = 5) by gavage with 25 cysts. After 24 hr, treatments with a daily dose of estragole (100 mg/kg) or thymol (80 mg/kg) were performed for 6 days (Oliveira et al., 2014). The concentrations of the compounds were selected on the basis of those used by Mota et al. (2012) that were effective against the *Plasmodium* spp. Negative control groups were treated with saline solution. Mice mortality was monitored daily for 30 days or until all animals were dead or presenting clinical signs of pain or distress as described above. At the end of the observation period and in cases of pain or suffering, the animals were humanely euthanized as described above. Surviving animals were euthanized, and their brains removed and homogenized in PBS to search for tissue cysts.

**Cytokine measurements**

For cytokine measurements, 4 female C57BL/6 mice were infected or not and distributed in groups (n = 4/cage) according to the following treatment schemes: NI/NT; I/NT; infected and treated with thymol, 80 mg/kg (I/T); infected and treated with estragole, 100 mg/kg; and infected and treated with sulfadiazine, 200 mg/kg (I/S).

**Ethics statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Brazilian Sanitary Vigilance Council put forward in resolution number 90/2004 and using Guidelines for Ethical Conduct in the Care and Use of Animals from the Federal University of Rio Grande do Norte (permit number: 46/2013).

**Anti-Toxoplasma gondii IgM and IgG ELISA**

Serum anti-*T. gondii* IgG and IgM antibody concentrations were measured by ELISA (Alvarado-Esquivel et al., 2011). Ninety-six-flat-bottom-well microtiter plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with wells containing *T. gondii* lysate antigen in 50 mM pH 9.6 sodium carbonate buffer at a final concentration of 1 μg/ml and volume of 100 μl were incubated overnight at 4 °C. The plates were then washed 4 times with pH 7.4 PBS containing 0.05% Tween 20 (PBS-T).

The plates were blocked with 200 μl of 2% nonfat powdered milk solution (Molico-Nestlé®, São Paulo State, Brazil) for 1 hr at 37 °C. Then 1/200 dilutions of serum samples in PBS (200 μl/well) were added to wells and incubated for 1 hr at 37 °C. After washing the plates 4 times with PBS-T, 100 μl of rabbit anti-mouse IgG or IgM-horseradish peroxidase (Sigma-Aldrich), diluted 1:10,000 (anti-IgG) or 1:1,000 (anti-IgM) in PBS, respectively, were added to each well. After 1 hr at 37 °C, plates were washed 5 times with PBS-T. Thereafter, plates were incubated for 10 min at room temperature with 50 μl/well of conventional chromogenic substrate 3,3,5,5’-tetramethylbenzidine (Intronig-Life Technologies, Gaithersburg, Maryland). The reaction was stopped by adding 30 μl/well of 4 N H₂SO₄ and absorbance was read at 450 nm.

**Infection during pregnancy as a model of congenital infection**

Virgin female Swiss Webster mice (n = 3) were placed in a male in a breeding box. The first day of pregnancy was identified by the presence of a vaginal plug or sperm in the vaginal smear of the female. After mating, the females were separated and 10 days postcoitum females were orally infected (Costa et al., 2009) with 10 ME49 strain cysts as previously described (Hermes et al., 2008). Starting 24 hr postinfection, pregnant females were orally (Epo) or subcutaneously (Esc) treated with estragole (100 mg/kg per day) or subcutaneously (Tsc) or orally (Tpo) treated with thymol (80 mg/kg per day) for 6 consecutive days. Infected, noninfected pregnant females (NI/NT) were used as control groups and received just the vehicle of the compounds. Females were separated before giving birth to offspring to avoid cross-fostering (i.e., each female provided alone for its offspring). The number of live pups was recorded, and these were monitored for 30 days. The pups were weighed on the 1st and 30th day after birth.

**Cytokine activity of the compounds in vivo**

Congenital infection in vivo and offspring analysis

Infection during pregnancy was used as a model of congenital infection. Virgin female Swiss Webster mice (n = 3) were placed in a male in a breeding box. The first day of pregnancy was identified by the presence of a vaginal plug or sperm in the vaginal smear of the female. After mating, the females were separated and 10 days postcoitum females were orally infected (Costa et al., 2009) with 10 ME49 strain cysts as previously described (Hermes et al., 2008). Starting 24 hr postinfection, pregnant females were orally (Epo) or subcutaneously (Esc) treated with estragole (100 mg/kg per day) or subcutaneously (Tsc) or orally (Tpo) treated with thymol (80 mg/kg per day) for 6 consecutive days. Infected, noninfected pregnant females (NI/NT) were used as control groups and received just the vehicle of the compounds. Females were separated before giving birth to offspring to avoid cross-fostering (i.e., each female provided alone for its offspring). The number of live pups was recorded, and these were monitored for 30 days. The pups were weighed on the 1st and 30th day after birth.

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All surgery was performed under ketamine hydrochloride plus xylazine anesthesia, and all efforts were made to minimize suffering.

**Statistical analysis**

GraphPad 5.0 software was used for graphical design (GraphPad Software, La Jolla, California). Quantitative variables were summarized as means and standard errors. An intragroup comparison was performed between values before and after treatment using a paired t-test. One-way ANOVA for multiple comparisons was carried out using GraphPad 5.0 software. Differences were considered significant when $P < 0.05$ and highly significant when $P < 0.001$.

**RESULTS**

**Cytotoxicity assay**

The cytotoxicity assay was performed using HepG2 and HeLa cell lines and murine peritoneal macrophages to evaluate the effects of thymol and estragole on cell viability. Dose-dependent inhibition of cell growth was observed in general for both compounds. In general, thymol exhibited greater inhibition than estragole in all cell lines. For thymol, $50\%$ cell growth inhibition (IC$_{50}$) values were 218.3, 182, and 92.1 $\mu$g/ml against HepG2, HeLa, and peritoneal macrophages, respectively, and for estragole, these values were 2,280, 2,684, and 267 $\mu$g/ml, respectively (Fig. 1).

**In vivo toxicity and anti-Toxoplasma activity**

Estragole and thymol exhibited low or moderate acute toxicity in vivo at single doses of up to 5.0 g/kg. Single doses of 0.312 and 1.25 g/kg of thymol and estragole, respectively, produced mainly ruffled fur. Death of some mice was observed just at single doses of 0.312 and 5.0 g/kg for thymol and estragole (Table I).

Regarding the survival of mice infected with ME49 strain, the treatments showed differences in mortality among S and I/NT or Tpo groups. The Epo, Esc, and Tsc groups exhibited similar survival when compared with NI/NT groups (Fig. 2). This result shows the anti-Toxoplasma activity of the Epo, Esc, and Tsc therapeutic regimen in maintaining the survival of infected animals.

<table>
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<tr>
<th>Compounds</th>
<th>Gender</th>
<th>D/T*</th>
<th>Days after administration</th>
<th>Signs</th>
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<tr>
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<td>—</td>
<td>Ns</td>
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<tr>
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<td>Female</td>
<td>0/6</td>
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<td>2 and 3</td>
<td>Piloerection, tremors, and death</td>
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<tr>
<td>78.12</td>
<td>Female</td>
<td>0/6</td>
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<td>Ns</td>
</tr>
<tr>
<td>312.5</td>
<td>Female</td>
<td>1/6</td>
<td>2 and 3</td>
<td>Piloerection, tremors, and death</td>
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<tr>
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<td>2–4</td>
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<tr>
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<td>1</td>
<td>Piloerection, tremors, and death</td>
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<tr>
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<td>Female</td>
<td>4/6</td>
<td>2</td>
<td>Piloerection, tremors, and death</td>
</tr>
</tbody>
</table>

* D/T, number of dead mice/treated mice; Ns, no symptoms of toxicity during the observation period.

ANOVA for multiple comparisons was carried out using GraphPad 5.0 software. Differences were considered significant when $P < 0.05$ and highly significant when $P < 0.001$.

**TABLE I. Effects of acute toxicity in treated mice (n = 6) with thymol, estragole, or vehicle control in a single dose.**

![Graphs showing cytotoxicity assay](https://bioone.org/journals/Journal-of-Parasitology on 30 Sep 2019)

**FIGURE 1. Influence of thymol (T) and estragol (E) on HepG2 (A), HeLa (B), and macrophage cell viability (C) after treatment for 24 hr. Results are expressed as percentages on the basis of nontreated controls. Three independent experiments were performed in triplicate.**
Animals treated with thymol and estragole showed a tendency to decrease the number of brain cysts when analyzed 30 days after infection (Fig. 3). However, the difference was not statistically significant when compared with the I/NT group. The I/S group showed a significant difference when compared with all other groups (Fig. 3). All animals exhibit cumulative change in body weight in the initial weeks of infection. Mice treated with thymol exhibited variations in body weight similar to those observed in the I/S group (Fig. 4) during 30 days postinfection by the \textit{T. gondii} ME49 strain.

**Effects of the compounds on congenital toxoplasmosis infection**

The effects of estragole and thymol in the congenital toxoplasmosis model were evaluated through analysis of the surviving offspring. All surviving pups were weighed on the first (Fig. 5A) and 30th day (Fig. 5B) after birth. Offspring of the NI/NT group showed greater body weight than all other groups on day 1. Body weight of the offspring from the Esc group (1.51 ± 0.04 g, n = 27) had no statistical difference when compared with...
that of the pups of the NI/NT group (1.68 ± 0.04 g, n = 19) and was significantly high compared with that of the pups of the I/NT group (1.31 ± 0.03 g, n = 31, P < 0.01). The body weight of the pups of the Epo (1.44 ± 0.02 g, n = 14) and Tsc (1.19 ± 0.02 g, n = 14) groups exhibited averages similar to the average weight of the offspring of the I/NT group and significantly less than the body weight of the offspring of the NI/NT group (Fig. 5A). The females of the I/Tpo group did not generate offspring (Fig. 5A).

The weight of pups 30 days after birth was significantly higher in those born to the Esc (14.9 ± 0.42 g, n = 27, P < 0.01) and Tsc (16.86 ± 0.64 g, n = 9) groups compared with those born in the I/NT group (12.53 ± 0.55 g, n = 26), and significantly different compared with pups from the NI/NT group (22.86 ± 0.68 g, n = 12) (Fig. 5B).

Cytokine measurements

Serum of mice from S, Tpo, Epo, and I/NT groups was assessed for detection of IL-10 and IL-12. The S-group mice exhibited lowest levels of detectable serum cytokines, whereas I/NT mice exhibited highest production of both cytokines. The Epo group demonstrated production of high levels of IL-12 (Fig. 6A), lowest levels of IL-10 (Fig. 6B), and high levels of INF-γ (Fig. 7), thus resulting in a high IL-12/IL-10 ratio (Fig. 6C). The Tpo-group mice induced intermediate levels of IL-10 and IL-12 in infected mice compared with the I/NT group (Fig. 6).

Anti-Toxoplasma gondii IgM and IgG ELISA

Another perspective on immunological evaluation consisted of measuring the production of IgG and IgM antibodies in different infected groups (Tpo, Epo, and S groups) (Alvarado-Esquivel et al., 2011). After infection, there was a significant increase in specific anti-T. gondii IgG in all groups (Fig. 8B). However, mice from the S group showed significantly lower IgG levels than those of the I/NT group at 14 and 30 days after infection. IgM measurements increased between the first and 14th day after infection in all groups, and remained at similar levels until the 60th day after infection (Fig. 8A). A significant decrease in IgM concentration was observed on the 14th day in the I/S group compared with with I/NT group. The Tpo group exhibited a significant increase in IgM concentration on the 14th day compared with the NI/NT group.

DISCUSSION

Previous results showed that thymol and estragole exhibit no cytotoxicity (IC50 ≥ 500 μg/ml after 24 hr against HeLa cells and macrophages) (Mota et al., 2012). The results of the present study showed that these compounds displayed no significant cytotoxicity toward HeLa, HepG2, or peritoneal macrophage cells after 24 hr. Muller et al. (1994) showed that estragole was not positive in a chromosomal aberration test with V79 cells or after direct treatment with rat liver mix or rat hepatocytes as source of metabolism. In this study, estragole was capable of inducing DNA repair in primary rat hepatocytes in vitro and in the liver in vivo in the unscheduled DNA synthesis test. Thymol cytotoxicity
has been studied by Llana-Ruiz-Cabello et al. (2014), who demonstrated no cytotoxic effects for thymol at any concentration and time of exposure. Ultrastructural changes evidenced by cellular damage such as lipid degeneration, mitochondrial damage, and nuclear fragmentation occurs, but only after thymol exposure at the highest concentration assayed (Llana-Ruiz-Cabello et al., 2014). Thus, knowledge of the cytotoxicity provided a basis for further evaluations in animals.

In vivo acute toxicity assays showed low or moderate toxicity. Estragole was found to have little toxicity, as observed by Gori et al. (2012). Thus, the data presented here provide some rational evidence to support further studies. The results suggest that these compounds can be safely used in animal tests.

Anti-protozoal activity was detected against the ME49 strain. The animal survival rate and the cumulative change of body loss responses (Munoz et al., 2011). Notoriously, cytokine IL-12 regulates nitric oxide synthesis through IFN-γ (Pifer and Yarovsky, 2011). In the Toxoplasma-host interaction, nitric oxide production is regulated by the partial inhibition of the synthesis of nitric oxide synthetase (Seabra et al., 2002). Potentially, cytokine modulation is highly important since nitric oxide is a part of the primary effectors in the immune system response against Toxoplasma gondii. Considering the immunomodulatory effects, cytokine IL-12 could protect the protozoans and prevent the action of oxidative stress caused by the host’s cellular immune response (McCarthy and Davis, 2003). However, thymol presents intriguing anti-T. gondii effects perhaps by acting on free radicals arising from tissue damage caused by the disease.

Estragole showed low toxicity and superior anti-Toxoplasma activity to thymol for both routes of administration, suggesting that the administration route does not interfere with biological activity. Other authors demonstrated effects of essential oil rich in estragole on the inhibition of growth of the protozoan Trypanosoma cruzi (Escobar et al., 2010b). However, there are no previously published reports regarding the effects of the other monoterpenes, such as thymol, against the ME49 strain of T. gondii in a murine model.

These compounds increase the survival of infected animals possibly also due to their anti-inflammatory activity, which has been the subject of previous reports (Ponte et al., 2012; Riella et al., 2012). Thus, estragole may exert a dual effect to extend the life of animals. Also, thymol exhibits high antioxidant activity, which could protect the protozoans and prevent the action of oxidative stress caused by the host’s cellular immune response (McCarthy and Davis, 2003). However, thymol presents intriguing anti-Toxoplasma effects perhaps by acting on free radicals arising from tissue damage caused by the disease.

Similarly, in a study on Artemisia annua tea infusions (Oliveira et al., 2009), a similar effect was observed when compared with the reference drug, sulfadiazine, in reducing morbidity and number of brain cysts.

The ME49 strain is usually a good alternative to in vivo anti-Toxoplasma activity tests (Martins-Duarte et al., 2010). It is a less virulent strain that facilitates the chronic phase of infection (Costa-Silva and Pereira-Chioccola, 2010). This allows more time to assess different treatment schemes. It is an especially good model because it better simulates the course of T. gondii infection in the general human population.

The effect of thymol, isolated from L. sidoides, has been demonstrated against the promastigote form of Leishmania amazonensis (Medeiros et al., 2011). Antiproteozaal activity also was observed against Trypanosoma cruzi (Santoro et al., 2007; Escobar et al., 2010a). Previous studies demonstrated anti-Toxoplasma activity versus the PRU strain of T. gondii using a phenol-terpenoid (carvacrol) compound (Dahbi et al., 2010). However, there are no previously published reports regarding the effects of the other monoterpenes, such as thymol, against the ME49 strain of T. gondii in a murine model.
involving parasite–host interactions given that the parasite is capable of modulating the immunological response of the host (Carruthers and Suzuki, 2007).

Thymol and estragole do not reduce the production of antibodies IgM and IgG; a significant decrease was observed only in the sulfadiazine treatment, as has been noted by others (Alvarado-Esquivel et al., 2011). Recently, T. gondii-specific antibody was shown to prevent cellular invasion and to limit systemic dissemination of tachyzoites during early acute T. gondii infection. Thus, different antibody classes appear to contribute to protection against the infection (Munoz et al., 2011). Thereby, the alterations in levels of the antibodies in sulfadiazine treatment reveal a disadvantage of standard treatment compared with other proposed treatments, especially in congenital toxoplasmosis. A possible explanation for sulfadiazine effects is related to the ability to induce hematological damage (Leport et al., 1988; Kaye, 2011).

In the present study, the therapeutic efficacy of thymol and estragole compounds was determined in the congenital model of infection. In this case, the infection was in an intermediate stage of pregnancy. Subcutaneous estragole treatment resulted in increased weight in newborns when compared with untreated controls or oral estragole treatment. In the 30 days after birth, all animals present a significant gain in body weight except for orally administered treatments. These data suggest that the estragole treatment outcome decreases the fetal exposure to T. gondii or inflammatory damage of toxoplasmosis in offspring, perhaps by decreasing intrauterine fetal growth. This scheme of congenital infection demonstrates greater efficiency in reproducing what happens to human beings, and the results are similar to another study (Wang et al., 2011). The results show that animals treated with the estragole improved their weight gain during development compared with the group of animals born to infected, untreated progenitor mice. This result deserves attention since many of the significant symptoms of congenital toxoplasmosis manifest during childhood of infected humans (Dubey and Jones, 2008; Shet, 2011).

On the basis of acute toxicity tests, we conclude that thymol and estragole are safe for use in animals. These treatments affect the cellular and humoral immune responses in mice infected with T. gondii and may increase the survival of these animals. We also observe an improvement in the living conditions of newborn and young pups of infected female mice. Moreover, further investigations are needed to evaluate the mechanism by which these compounds alter the parasite–host interaction and to assess the influence of these compounds on specific targets of the metabolic pathways of T. gondii, as a perspective to develop new drugs for the treatment of toxoplasmosis.

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LITERATURE CITED


