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ANTI-*TOXOPLASMA* ACTIVITY OF ESTRAGOLE AND THYMOL IN MURINE MODELS OF CONGENITAL AND NONCONGENITAL TOXOPLASMOSIS

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ABSTRACT: Toxoplasmosis is caused by *Toxoplasma gondii*, an obligatory intracellular protozoan. Normally benign, *T. gondii* infections can cause devastating disease in immunosuppressed 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 commercial estragole and thymol. The congenital experimental model was used with intermediate stages of maternal infection. The serum levels of immunoglobulin (Ig)M, IgG, interleukin (IL)-10, IL-12, and interferon-gamma (IFN-γ) were quantified from infected and treated C57Bl/6 mice. Estragole and thymol respectively exhibited low to moderate in vivo toxicity and cytotoxicity. Animals treated with estragole showed high IFN-γ and strong type 1 helper T cell response. Both compounds were active against *T. gondii* ME49 strain. Furthermore, orally administered estragole in infected pregnant mice improved the weight of offspring compared with untreated controls. Subcutaneous administration of both compounds also increased the weight of mouse offspring born to infected mothers, compared with untreated controls. Estragole and thymol display important anti-*Toxoplasma* activity. Further studies are needed to elucidate the mechanism of action 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 (Değerli 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 "alecrim-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 antiplasmodial 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 1 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

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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 peritoneal 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% CO_2 at 37 C and were subcultured every 7 days.

Estragole was dissolved (1.0%) in dimethyl sulfoxide (DMSO; Sigma-Aldrich), whereas thymol was dissolved in distilled water. The resulting solutions were serially diluted with fresh DMEM. Then, the dilute sample solutions were applied at final concentrations of 78.3–5,000 μg/ml (7.83–500 μg/ml to peritoneal murine macrophages) to wells each containing 1×10^4 cells in 96-well microplates. The plates were incubated in a final volume of 200.0 μl at 37 C for 24 hr. Control cells were incubated in the presence of 1% DMSO in drug-free medium. The viability of the cells was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay (Oliveira et al., 2014). Briefly, MTT (0.5 mg/ml) was dissolved in phosphate-buffered saline (PBS) and filtered through a 0.22-μm membrane and then 100 μl/well was added to a 96-well plate and incubated at 37 C for 3 hr. Formazan extraction was performed with DMSO (200 μl/well) at 25 C and absorbance was read at 570 nm on an ultraviolet–visible spectrophotometer.

Toxicity tests

The acute toxicity assay was performed before the study in the congenital toxoplasmosis model. Compounds were orally administered with a single dose of the compounds (doses of 19.5 to 5,000 mg/kg [n = 6 animals]) or vehicle (DMSO 1% for the estragole or just distilled water for the thymol). Mice were monitored daily for symptoms of suffering and the survival and cumulative mortality during 30 days. Mice with apparent presence of clinical pain (vocalization, guarding affected body parts, or self-mutilation) or signs indicative of death before the planned end of the experiment (for example: inability to reach water or food or when moribund state) or death is expected before the next planned time of observation (for example: mice present convulsions, recumbency, or tremor) were anesthetized with ketamine hydrochloride (Ketamina®, Agener União, União Química, São Paulo State, Brazil) and xylazine (Calmium®, Agener União) intraperitoneally and then humanely killed by cervical dislocation. Surviving animals after 30 days were also anesthetized and then euthanized as described above.

Anti-*Toxoplasma* 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. Cysts were quantified in brain suspensions, and after standard dilution, each mouse was infected with cysts 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) or sulfadiazine (S group, 200 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.

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 with 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, nontreated pregnant females (I/NT) and non-infected, non-treated 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 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).

Interleukin (IL-12 and IL-10) levels in serum samples were quantified by the so-called sandwich enzyme-linked immunosorbent assay (ELISA) performed using the manufacturer's instructions (BD Biosciences, San Jose, California) and compared with a standard curve built with the respective recombinant murine cytokine. The cytokine detection limit was 15.6 pg/ml in both assays. A BD™ mouse typ1/type2 helper T cell (Th1/Th2) cytokine cytometric bead array kit (BD Biosciences) was used to quantify interferon-gamma (IFN-γ) in serum previously obtained from C57BL/6 strain mice using the manufacturer's instructions.

C57BL/6 strain mice were infected with 10 cysts. After 24 hr, infected mice were treated per gavage with a daily dose of estragole (100 mg/kg) or thymol (80 mg/kg) during 6 consecutive days. The animals were observed daily for 60 days. On days 7, 14, 28, and 60 postinfection, animals were anesthetized, and blood samples were collected from the orbital plexus. After all the punctions, the surviving animals were given a lethal dose of the anesthetics ketamine hydrochloride and xylazine. The samples were centrifuged, and the supernatant serum was stored for determination of cytokine levels and immunoglobulin M and G (IgM and IgG, respectively) antibodies.

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 (Invitrogen-Life Technologies, Gaithersburg, Maryland). The reaction was stopped by adding 30 μl/well of 4 N H_2SO_4 and absorbance was read at 450 nm.

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).

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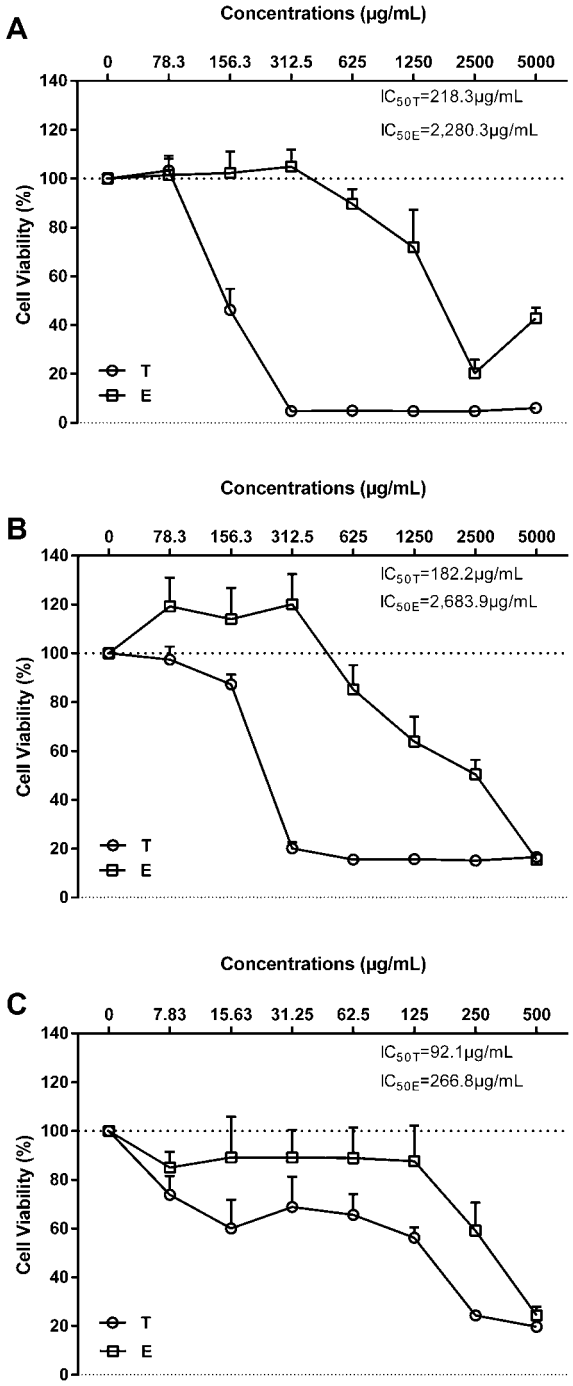


FIGURE 1. Influence of thymol (T) and estragol (E) on HepG2 (**A**), HeLa (**B**), and macrophage cell viability (**C**) after treatment for 24 hr. Results are expressed as percentages on the basis of nontreated controls. Three independent experiments were performed in triplicate.

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 $P < 0.001$.

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

Compounds (mg/kg)	Gender	D/T*	Days after administration	Signs
Thymol				
Vehicle	Female	0/6	—	Ns
19.5	Female	0/6	—	Ns
78.12	Female	0/6	—	Ns
312.5	Female	1/6	2 and 3	Piloerection, tremors, and death
1,250	Female	2/6	2–4	Piloerection, tremors, and death
5,000	Female	6/6	1	Piloerection, tremors, and death
Estragole				
Vehicle	Female	0/6	—	Ns
19.5	Female	0/6	—	Ns
78.12	Female	0/6	—	Ns
312.5	Female	0/6	—	Ns
1,250	Female	0/6	2–4	Piloerection
5,000	Female	4/6	2	Piloerection, tremors, and death

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

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 inhibiton (IC_{50}) values were 218, 182, and 92.1 µg/ml against HepG2, HeLa, and peritoneal macrophages, respectively, and for estragole, these values were 2,280, 2,684, and 267 µg /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.

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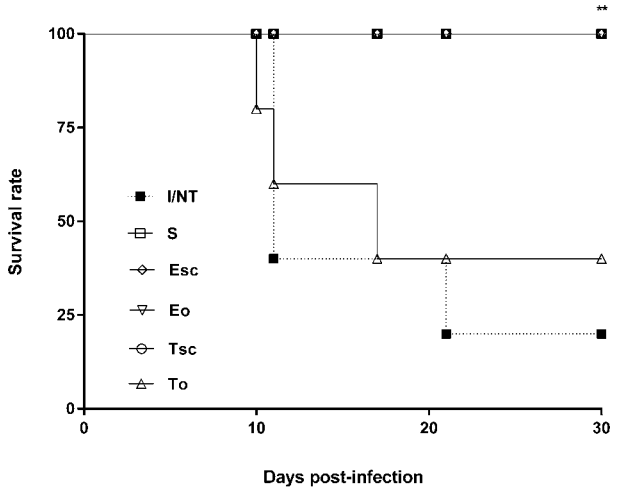


FIGURE 2. Survival rate of Swiss female mice after infection with the *Toxoplasma gondii* ME49 strain. I/NT, infected, nontreated group; S, sulfadizine treatment group; Esc, estragole subcutaneous treatment group; Eo, estragole oral treatment group; Tsc, thymol subcutaneous treatment group; To, thymol oral treatment group.

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 *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

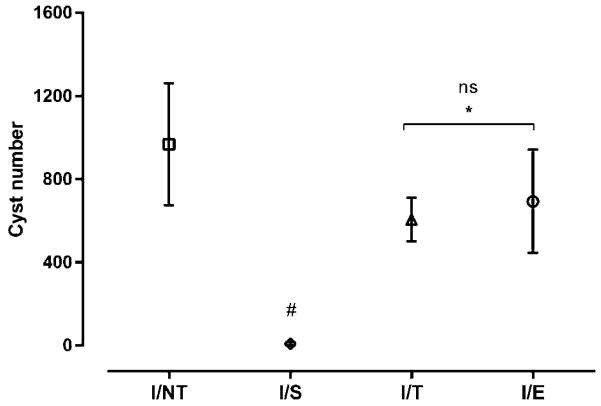


FIGURE 3. Number of brain cysts in C57BL/6 mice orally infected with 10 *Toxoplasma gondii* ME49 strain cysts. I/NT, infected, nontreated group; I/T, infected thymol-treated group; I/E, infected estragole-treated group; I/S, infected sulfadiazine-treated group (#, $P < 0.05$ compared with all others groups; *, $P < 0.05$ compared with I/S group; ns, nonsignificant compared with I/NT group).

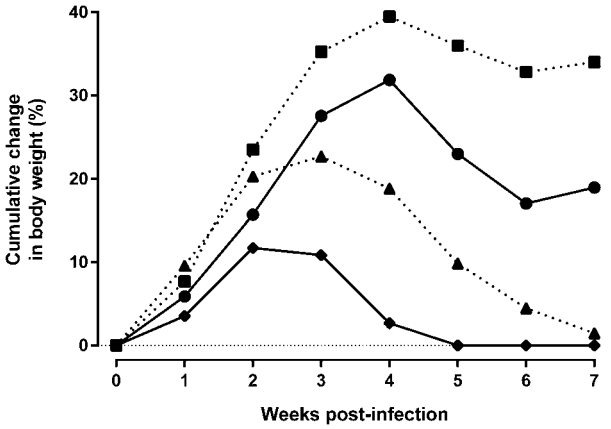


FIGURE 4. Percentage of cumulative body weight in C57BL/6 mice orally infected with 10 *Toxoplasma gondii* ME49 strain cysts (■, infected, nontreated group; ▲, infected thymol-treated group; •, infected estragole-treated group; ◆, infected sulfadiazine treated group).

(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

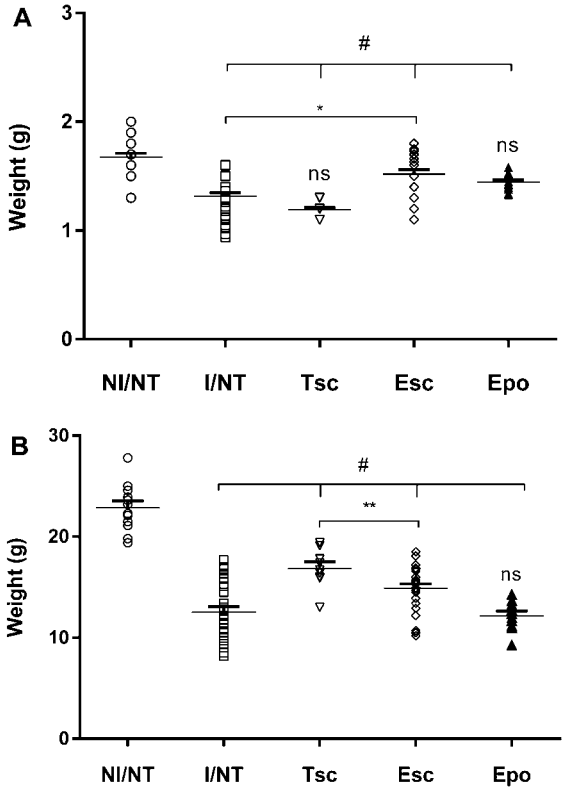


FIGURE 5. Weight of pups from mothers infected with 10 *Toxoplasma gondii* ME49 strain cysts and submitted to different treatments on days 1 (**A**) and 30 (**B**) after birth (NI/NT, noninfected, nontreated group; I/NT, infected, nontreated group; Esc, infected subcutaneous estragole-treated group; Epo, infected oral estragole-treated group; Tsc, infected subcutaneous thymol-treated group) (#, significant compared with NI/NT group; *, significant compared with I/NT group; ns = nonsignificant compared with I/NT group).

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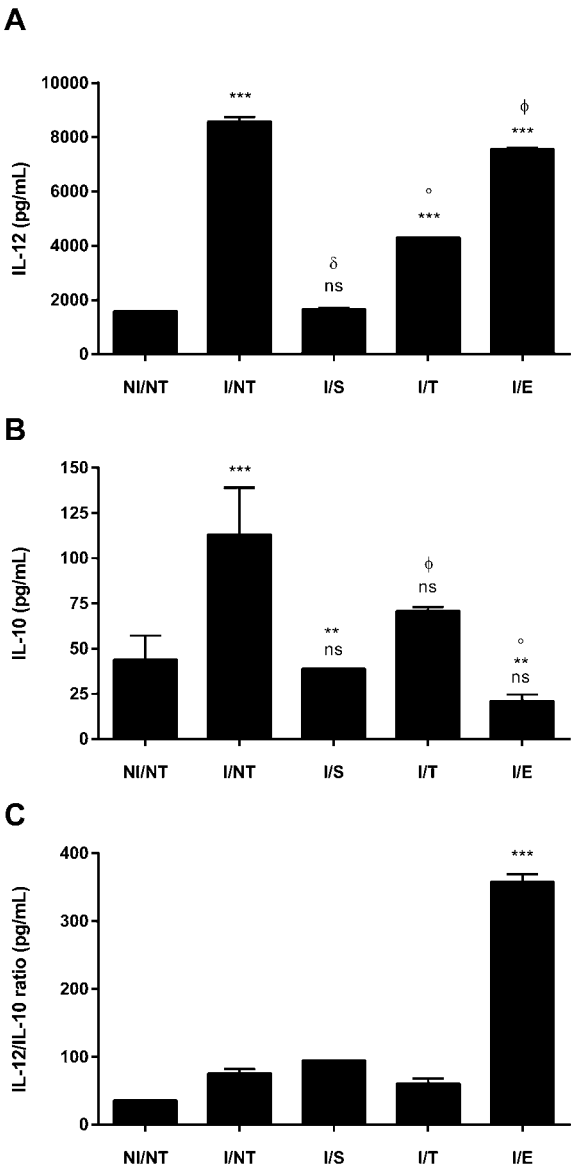


FIGURE 6. Serum cytokine balance induced by the different treatments in C57BL/6 mice infected with 10 *Toxoplasma gondii* ME49 strain cysts. Production of IL-10 (**A**) and IL-12 (**B**) and IL-12/IL-10 ratio (**C**) in C57BL/6 mice infected with 10 *T. gondii* ME49 strain cysts (***, $P < 0.001$ compared with NI/NT group; δ, $P < 0.001$ compared with I/NT group; ○, $P < 0.01$ compared with I/NT group; Φ, $P < 0.05$ compared with I/NT group).

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

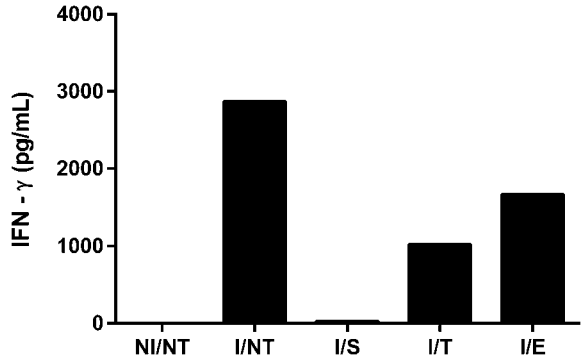


FIGURE 7. Serum interferon-γ induced by different treatments in C57BL/6 mice infected with 10 *Toxoplasma gondii* ME49 strain cysts.

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 to 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 ($IC_{50} \geq 500$ 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

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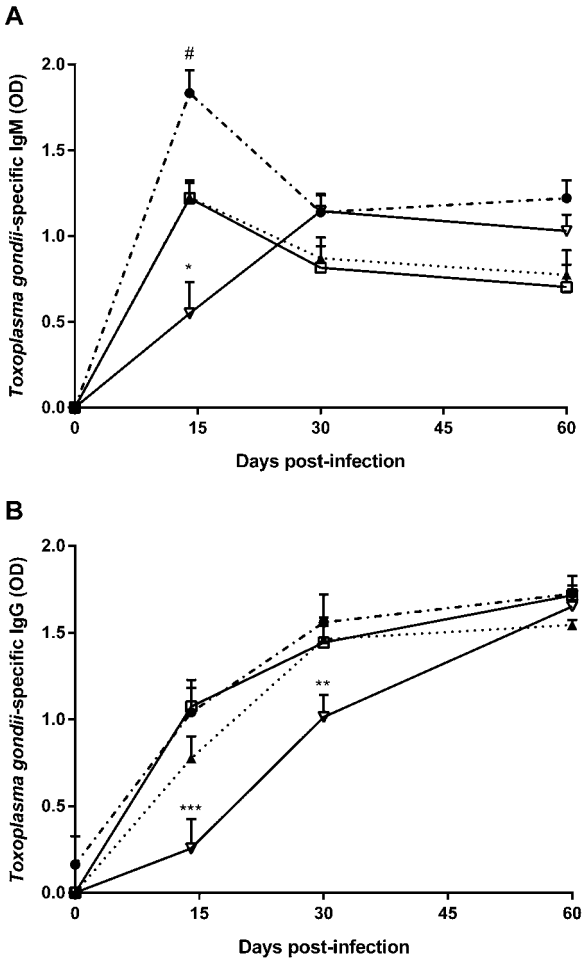


FIGURE 8. Serum immunoglobulin level induced by the different treatments (•, infected thymol-treated group [I/T]; □, infected estragole-treated group [I/E]; ▲, infected, nontreated group [I/NT]; ◇, infected sulfadiazine-treated group [I/S]). Production of *Toxoplasma gondii*-specific IgM antibodies (**A**) and *T. gondii*-specific IgG antibodies (**B**) from infected C57BL/6 mice with 10 *T. gondii* ME49 strain cysts (***, $P < 0.001$ compared with all other groups on the 15th day; **, $P < 0.01$ compared with all other groups on the 30th day; *, $P < 0.05$ compared with I/NT group on the 15th day; #, $P < 0.05$ compared with all other groups on the 15th day; ns = no significance compared with I/NT).

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-protozoan activity was detected against the ME49 strain. The animal survival rate and the cumulative change of body loss demonstrated antiprotozoal activity for thymol by the subcutaneous route and estragole by the oral and subcutaneous routes. 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). Antiprotozoal 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.

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, this is the first report on the biological activity of estragole in a murine model of infection with *Toxoplasma gondii*.

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.

Considering the immunomodulatory effects, cytokine IL-12 regulates nitric oxide synthesis through IFN-γ (Pifer and Yarovinsky, 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 *T. gondii* (Brunet, 2001).

Previous results obtained by this group demonstrated an immunomodulatory effect for estragole (data not shown). This compound induces the production of nitric oxide in macrophages. Herein, estragole treatment in infected mice induced high levels of Th1 response (higher IL-12 levels and lowest IL-10). This result is in agreement with an independent report (Liu et al., 2006). Estragole-treated mice also had the highest IFN-γ rate among the treated groups. This result is relevant considering that IFN-γ is a fundamental part in organizing a strong Th1 response, highly effective against the parasite. This immune response limits tachyzoite replication and drives the generation of specific T-cell responses (Munoz et al., 2011). Notoriously, *Toxoplasma* has an immunomodulatory ability particularly important in research

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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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