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Effects of *Thymus vulgaris* ethanolic extract on chronic toxoplasmosis in a mouse model

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Abstract The current work was undertaken to investigate the potential effectiveness of *Thymus vulgaris* ethanolic extract (*TVE*) against *Toxoplasma gondii* infection in chronic experimental toxoplasmosis. To evaluate prophylactic effects, mice received 500 mg/kg *TVE* for 5 days before they were infected by an avirulent Me49 *T. gondii* strain. To investigate the therapeutic effects of the extract postinfection, daily treatment with *TVE* was initiated at 6 weeks postinfection and continued for 10 days. The following groups of animals were used as controls: uninfected/non-treated, infected/non-treated, and infected/treated with a combination of pyrimethamine and sulfadiazine. Brain cyst count and histopathological changes using H&E and Feulgen stains were used to evaluate the efficacy of *TVE*. The mean number of brain cysts was significantly decreased by 24 % in mice treated prophylatically with *TVE*. *TVE* also significantly reduced the mean number of brain cysts when administered to animals already chronically infected with *T. gondii*. The effect of *TVE* was comparable to that of treatment with a mixture of sulfadiazine and pyrimethamine (46 and 51 % reduction, respectively). Moreover, considerable amelioration of the pathological lesions in the brain and retina was observed. The results demonstrate the potential efficacy of *T. vulgaris* as a new natural therapeutic and prophylactic agent for use in the treatment of chronic toxoplasmosis.

Keywords *Thymus vulgaris* · Therapeutic · Prophylactic · Toxoplasmosis · In vivo

Introduction

Toxoplasmosis is caused by the obligatory intracellular coccidian protozoan, *T. gondii*. Although approximately 30 % of the world population suffer from *T. gondii* infection and harbor cysts in the brain, overt disease symptoms such as encephalitis are only evident during immune suppression (Pusch et al. 2009).

Treatment of toxoplasmosis is essential as *T. gondii* infection may be complicated by serious morbidity and mortality, particularly in pregnant women and in immunocompromised patients. Pyrimethamine-sulfadiazine combination remains the gold standard for treatment of and prophylaxis against toxoplasmosis. However, this therapeutic regimen is not always suitable for prolonged treatment of toxoplasmosis because of the great possibility of appearance of adverse side effects and the potential to contribute to clinical failure by selecting for drug-resistant parasite variants (Hong et al. 2014). Moreover, use of pyrimethamine for treatment of *T. gondii* infection is associated with suppression of bone marrow and may result in neutropenia even when accompanied by leucovorin supplements. In addition, this combination may give rise to serious allergic reactions and hepatic and renal complications (Mui et al. 2005). Furthermore, this treatment is not used to treat congenital toxoplasmosis in the first trimester of gestation when folate depletion may have additional detrimental consequences for early fetal development. Consequently, new effective therapies with less severe side effects are critically needed (Ferreira et al. 2006).

Some plant extracts and chemical substances isolated from these plants exhibit anti-helmenthic activity and therefore...
offer an alternative that provides both sustainable and environmentally acceptable therapy with less side effects (Elissondo et al. 2008; Edeoga et al. 2005).

*Thymus vulgaris* (thyme), a member of the family Lamiaceae, is a very popular traditional medicinal plant widely used in the Middle East as well as all over the world. This plant has many folkloric uses. *Thymus vulgaris* has antispasmodic, expectorant, anti-tussive, anti-broncholitic, analgesic, anti-inflammatory, anti-helmenthic, carminative, diuretic, and sedative properties (Nickavar et al. 2005).

Its active constituents responsible for pharmacological effects include essential oils (borneol, carvacrol, cymol, linalool, and thymol), tannin, flavonoids (apigenin and luteolin), saponins, and triterpenic acids (Ghazanfar 1994). Essential oils and extracts from fresh leaves and flowers of thyme can be used as aromatic additives in foods, pharmaceuticals, and cosmetics. Moreover, these essential oils were found to inhibit growth of a wide range of organisms that cause various diseases (Akin et al. 2010; Karata and Ertekin 2010). In this work, the possible efficacy of thyme in providing prophylaxis and treatment against *T. gondii* was investigated. The results show for the first time very promising effects of thyme in this regard and open a possibility of discovering other effective plants in the rich Egyptian botanical flora.

**Materials and methods**

**Drugs and plant materials**

The selection of thyme was made on the basis of information gathered about its use in traditional medicine. *T. vulgaris* (thyme) leaves were purchased from ISIS Company (Egypt) in October 2014. The plant was identified by the Department of Medicinal and Aromatic Plants, Horticulture Research Institute, Giza, Egypt, and voucher specimens were recorded for future reference. The leaves (500 g) were cleaned and shade dried, ground into powder using an electrical blender, and then homogenized in ethanol (95%; 500 ml). The mixture was left in a conical flask at room temperature for 3 days and then was filtered through a fine muslin cloth and filter paper (Whatman No. 1). The extract was concentrated using a rotary evaporator (Sigma-Aldrich, USA) and then lyophilized to yield *Thymus vulgaris* extract (*TVE*) (Attia et al. 2015).

Sulfadiazine (Dohms Laboratories) and pyrimethamine (Sigma Chemical Co., St. Louis, MO) were provided in powder form and prepared daily as liquid suspensions. The homogenized suspensions were administered orally to mice via a feeding tube. Infected mice were treated with a combination of sulfadiazine at a dose of 200 mg/kg/day and pyrimethamine at a dose of 12.5 mg/kg/day (Romand et al. 1993).

**Parasites and their maintenance**

Me49 avirulent *T. gondii* strain (kindly provided by the National Research Center, Giza, Egypt) was regularly maintained by repeated inoculation of Swiss albino mice every 8 weeks with 0.1 ml of brain homogenate of previously infected mice containing approximately 100 tissue cysts/ml to establish chronic toxoplasmosis (Djurkovic-Djakovic et al. 2002). Mice were infected orally by gavage with brain suspension containing *T. gondii* cysts (ten cysts/mouse). To prepare the brain suspension, 8-week infected mice were sacrificed, brains were removed, and small parts of the cerebrum were fixed in 10% formalin for histopathological study, while the remaining parts were prepared in a tissue homogenizer (Wheaton USA) with 1 ml saline each. For cyst enumeration, 0.1 ml of the brain suspension was placed on a slide and microscopically counted under a ×40 lens. The suspension was then diluted to a concentration of 100 cysts/ml (Djakovic and Milenkovic 2001).

**Experimental animals, infection, and treatment schedule**

To test the efficacy of *T. vulgaris* in a chronic model of experimental toxoplasmosis, a total of 50 laboratory-bred male Swiss albino mice were used (10 weeks old, weighing ∼40 g.) Animals were housed and maintained in a suitable rearing environment with free access to food and water throughout the experiment. Mouse stools were examined conventionally to exclude the presence of parasites (Garcia and Bruckner 1977). Mice were allocated into five main groups (ten mice each) as follows:

- **Group I:** noninfected mice
- **Group II:** chronically infected (infected with ten cysts/mouse by gavage), but not treated with TVE
- **Group III:** (pretreated or prophylactic group)—infected and received TVE in a dose of 500 mg/kg/day for 5 days before animal infection by *T. gondii* cysts
- **Group IV:** infected with *T. gondii* cysts and then treated with TVE (500 mg/kg/day for 10 days),
- **Group V:** infected and treated with a combination of sulfadiazine at a dose of 200 mg/kg/day and pyrimethamine at a dose of 12.5 mg/kg/day (Romand et al. 1993)

In groups IV and V, treatment started 42 days postinfection and continued for 10 days.

Animals of all groups were sacrificed 2 weeks after the end of therapy and their brains removed. Each brain was divided into two halves. One half was used for brain cysts count. The
other half was fixed in 10 % formalin for histopathological and histochemical studies.

Assessment of TVE efficacy

Parasitological evaluation

The number of Toxoplasma cysts was counted in ten high power fields (HPF) and then the mean number was estimated for each mouse followed by calculation of the mean numbers of cysts in each infected group (Djakovic and Milenkovic 2001).

Histopathological evaluation of the brain and retina

Parts of the brain (grey and white matter of the cerebrum) and retina of all studied groups were fixed in 10 % buffered formalin (pH 7.4) for 48 h. The tissue was dehydrated in an ethyl alcohol series, cleared in xylene and embedded in paraffin. The sections (5 μm) were de-waxed, hydrated, and stained in Mayer’s hemalum solution for 3 min. They were then stained with hematoxylin and eosin and prepared according to the method of Abdel Wahab et al. (1989).

Histochemical evaluation of the brain and retina using the Feulgen nuclear reaction for DNA

The Feulgen reaction allows DNA in situ to be specifically stained based on the reaction of Schiff or Schiff-like reagents with aldehyde groups engendered in the deoxyribose molecules by HCl hydrolysis. The staining intensity is proportional to the DNA concentration (Chieco and Derenzini 1999). Grades of apoptosis according to color changes of DNA were evaluated as faint staining of cell DNA (marked apoptosis), pale red staining of cell DNA (moderate apoptosis), less than bright red staining of cell DNA (mild apoptosis), and bright red staining of cell DNA (normal).

Statistical analysis

The collected data were analyzed by the Statistical Package for Social Science (SPSS) program, version 11.0 for Windows. Quantitative data were expressed as mean and standard deviation and analyzed using F-test (ANOVA) to compare the different studied groups.

Results

Estimation of Toxoplasma cyst count in the brain

The brain was chosen in the present study as an indicator of the severity of infection because chronic infection by T. gondii is characterized by cysts located predominantly in the central nervous system. In the group chronically infected with T. gondii but not treated with TVE, the mean number of T. gondii cysts was 630±58. Treatment of mice with TVE for 5 days before infection (prophylactic group) showed a statistically significant decrease of cyst burden (480±62). This represents a 24 % decrease in the cyst count as a result of TVE treatment (Table 1). TVE also reduced the cyst count in the brain when administered following chronic infection with T. gondii. In this experimental paradigm, TVE decreased the number of cysts to 340±26.14, a reduction of 46 %. In contrast, sulfadiazine-pyrimethamine treatment in the chronically infected mice decreased the cyst count by 51 %. Thus, neither TVE nor sulfadiazine-pyrimethamine treatments achieved complete eradication of Toxoplasma cysts. Under ordinary light microscopy, tissue cysts obtained from infected untreated controls were often spherical with well-defined intact cyst walls. They also varied in size, ranging from small cysts containing 1–2 bradyzoites to large cysts containing more than 50 bradyzoites. Cysts were much smaller, rounded, or oval with irregular cyst walls in animals treated with either TVE or sulfadiazine-pyrimethamine.

Histopathological and histochemical studies of the brain and retina

There were marked histopathological changes in the brain tissues of mice infected with T. gondii compared to the uninfected control mice (Fig. 1a). Brain sections from infected untreated mice showed well-defined Toxoplasma cysts in the meninges and cerebral tissue (white matter or at the junction of grey and white matter) and inflammatory infiltrates in the meninges (Fig. 1b). This inflammatory reaction was evident as a congestion of the meninges, with mononuclear cell invasion and necrosis of neurons. In the prophylactic group in which mice treated with TVE 5 days before infection by T. gondii cysts (Fig. 1c), histopathological examination revealed moderate pathological changes in the form of necrosis of neurons and neuronophagia, congestion of meningeal blood vessels associated with inflammatory infiltrate in the meninges, and focal aggregations of microglia cells. While in the TVE-treated group, there was a marked decrease in cyst numbers and even their disappearance in some sections associated with minimal pathological changes in the form of focal aggregations of glial cells (Fig. 1d). Similarly, animals treated with a combination of sulfadiazine and pyrimethamine showed focal aggregations of glial cells and focal cerebral necrosis (Fig. 1e).

Similar findings were observed in the retina, as there was amelioration of retinal pathology and rearrangement of retinal
Table 1  The mean number of Toxoplasma cysts in brains of mice with established chronic toxoplasmosis after treatment with TVE compared to untreated chronically infected controls and drug control group (treated by a combination of sulfadiazine and pyrimethamine)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Mean number <em>T. gondii</em> Cysts ± SD</th>
<th>% of Reduction</th>
<th>t test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninfected and untreated</td>
<td>0 ± 0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Infected and untreated</td>
<td>630 ± 58</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Infected and TVE treated (prophylactic)</td>
<td>480 ± 61</td>
<td>24</td>
<td>4.81b</td>
<td>0.001**</td>
</tr>
<tr>
<td>Infected and TVE treated</td>
<td>340 ± 26</td>
<td>46</td>
<td>17.11b</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Postinfection treated</td>
<td>310 ± 30.91</td>
<td>51</td>
<td>13.52b</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

* Compared to noninfected and untreated group
b Compared to infected and untreated group
c Compared to pyrimethamine-sulfadiazine treated group
** P<0.001: highly significant

Fig. 1  Histopathological sections of brain tissue showing: a Brain section from normal uninfected control mice (group I) showing preserved architecture. b Brain section from infected, untreated control group (II), showing severe necrosis of neurons. c Brain section from animals pretreated with TVE prior to infection with *T. gondii* (prophylactic group) (III), showing moderate necrosis of neurons and neuronophagia. d Brain section from infected animals that were later treated with TVE (IV), showing focal aggregations of microglia cells with moderate necrosis of neurons. e Brain section from infected, pyrimethamine-sulfadiazine treated group (V), showing aggregations of microglia cells with mild focal necrosis of neurons. (H&E ×400)
layers in all treated groups compared to infected untreated controls (Fig. 2a–d).

In the present work, the Feulgen histochemical stain was used to demonstrate DNA in tissue sections and for the assessment of apoptotic changes in brain and retinal tissues of treated and control mice. Normally, the nuclei of brain and retinal cells were stained bright red as observed in the normal control group (Figs. 3a and 4a). In the infected untreated control group, *T. gondii* induced marked cell apoptosis (Figs. 3b and 4b) in comparison with uninfected mice whose normal DNA showed bright red staining. In mice prophylactically treated with *TVE*, there was pale red staining of brain and retinal cell DNA indicating moderate apoptosis of these cells (Figs. 3c and 4c). Both groups treated with *TVE* or a combination of sulfadiazine and pyrimethamine starting 6 weeks postinfection showed less than bright red staining of brain DNA indicating mild apoptosis (Figs. 3d, e and 4d, e).

**Discussion**

Since ancient times, various herbal medicines have been tested to treat different parasites (Kanojiya et al. 2015). It was found that extracts of many herbal plants exhibit anti-*Toxoplasma* activity. These plants include *Myrrh* (AL-Zanbagi 2007), *Piper nigrum*, *Capsicum frutescens*, *Curcuma longa* (AL-Zanbagi 2009), *Zingiber officinale* (Choi et al. 2011), *Azadirachta indica* (neem) and *Melia azedarach* (Melo et al. 2011), and *Nigella sativa* (Rayan et al. 2011; Madi et al. 2016).

Antiparasitic activity of *TVE* against a variety of protozoa has been demonstrated by many researchers. EL-Sayed (2009), for example, reported that the ethanolic extracts of *T. vulgaris* have significant inhibitory effects on the growth of *Blastocystis hominis* cysts. Hydroalcoholic extract of *T. vulgaris* was also shown to be effective against the trophozoites of *Entamoeba*
Histolytica (Behnia et al. 2008). TVE was shown to be a growth inhibitor of Trypanosoma brucei (Mikus et al. 2000) and Acanthamoeba castellanii trophozoites and cysts (Polat et al. 2007), and showed moderate activity on Giardia lamblia (Calzada et al. 2006). In addition, Santoro et al. (2007) showed that thyme and thymol were active against epimastigotes and trypomastigotes of Trypanosoma cruzi. Also, they observed that thyme-treated epimastigotes and trypomastigotes showed cytoplasmic swelling with morphological alternations in the plasma and flagellar membrane and concluded that thyme’s essential oils permeate the cell membrane and kill parasites by affecting their cytoplasmic metabolic pathways or organelles which would result in cell membrane lysis. Moreover, Nilforoushzadeh et al. (2008) suggested that a hydroalcoholic extract of T. vulgaris was effective in the treatment of cutaneous leishmaniasis in mice and attributed this effect to stimulation of natural killer cells activity and release of nitric oxide and tumor necrosis factor from macrophages.

The present study investigated the effects of thyme in murine chronic infection with avirulent Me49 T. gondii strain, and the results suggest using thyme as a new potential therapy. These findings show that starting the administration of TVE extract for 5 days before infection by T. gondii cysts had a potent prophylactic effect against chronic toxoplasmosis. Moreover, oral administration of TVE 6 weeks postinfection revealed remarkable therapeutic effects against chronic toxoplasmosis as there was a highly significant decrease in the mean number of T. gondii cysts in the brains. The magnitude of this effect (46 %) was comparable to that of the standard treatment with sulfadiazine-pyrimethamine (51 %).
Various pharmacological activities of TVE may be attributed to its phytoconstituents, thymol and carvacrol. Though the exact mode of the antimicrobial actions of TVE is poorly understood, TVE has been shown to increase the immune responses and to exhibit potent antioxidant properties (Rota et al. 2007). Moreover, TVE has been shown to alter permeability of the cell membrane as well as to modulate membrane organization and surface electrostatics, leading to extracellular release of membrane-associated materials with subsequent parasite destruction (Sanchez et al. 2004). These suggested mechanisms of action of TVE are different from those proposed for sulfadiazine and pyrimethamine. The latter drugs are thought to act through inhibiting T. gondii folic acid synthesis, which is essential for parasite survival and replication (Doliwa et al. 2013).

The present study showed amelioration of many T. gondii-induced cerebral adverse effects by TVE. Brains of untreated infected mice showed marked inflammatory reactions, reflected mainly as proliferation of mononuclear cells, congestion of meninges with mononuclear cells invading the meninges, necrosis of neurons, and Toxoplasma cysts either in meninges or embedded in cerebral tissue. These observations are in agreement with findings published by other groups (Waree 2008). One of the main findings of the present study is a significant amelioration of these pathological lesions by TVE, both when administered as a prophylactic regimen or following infection with T. gondii. These effects may be due to the ability of TVE to stimulate natural killer cell activity and release of nitric oxide and tumor necrosis factor from macrophages (Nilforoushzadeh et al. 2008).
DNA damage in *T. gondii* infected mice observed in this and other studies may be attributed to the host immune response against infection. These responses include antibody production (humoral immune response) and activation of various defensive cellular mechanisms (Silva et al. 2002), for example liberation of nitric oxide, interferon-gamma (IFN-γ), tumor necrosis factor (TNF-α), and reactive oxygen species by macrophages (Kawazoe 2000). While such defensive mechanisms help to eradicate an invading parasite, they also result in genotoxic effects on DNA (Fitzpatrick 2001).

In this study, *T. gondii* induced changes. This effect may be attributed to the potent antioxidant properties of *TVE* that offer protection against reactive oxygen species (Rota et al. 2007). In addition, phenolic compounds in *TVE* exhibit considerable anti-microbial and anti-fungal activities through interference with cell metabolism (Ghazanfar 1994) and inhibition of protein or DNA synthesis (Chopra and Hacker 1992). Moreover, they have the ability to alter the permeability of cell membranes as well as the capacity to affect membrane organization and surface electrostatics. This leads to release of membrane-associated materials from the cells to the external medium and subsequent destruction of the pathogenic bacteria (Sanchez et al. 2004).

In conclusion, this study demonstrated, for the first time, the efficacy of a novel herbal therapy in the treatment of chronic toxoplasmosis and in providing prophylaxis against this parasite. The effects of this herbal medicine were comparable to those produced by the common treatment with a combination of pyrimethamine and sulfadiazine. Further studies are recommended in order to investigate the effectiveness of this new regimen against *T. gondii* under conditions known to reduce resistance to the parasite, e.g., immunosuppression. We also plan to study the effects of *TVE* in combination with other antiparasitic medications in order to optimize therapeutic efficacy and reduce side effects. Ultimately, we plan to investigate whether introduction of this herb as a routine food supplement could be beneficial in offering prophylaxis against *T. gondii* infection.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights informed consent** This study was approved by the Scientific Research Ethical Committee, Faculty of Medicine, Benha University. The experimental animal studies were conducted in accordance with international guidelines for animal care. All experimental mice were housed under standard laboratory conditions with an average temperature of 20–25 °C and were given drinking water and regular mouse diet.

**References**


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