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Effects of *Aloe vera* and *Eucalyptus* methanolic extracts on experimental toxoplasmosis *in vitro* and *in vivo*

Bahareh Mirzaalizadeh[a,b], Mehdi Sharif[a,c], Ahmad Daryani[a,d], Mohammad Ali Ebrahimzadeh[e], Mehryar Zargari[f], Shahabeddin Sarvi[a,d], Saeed Mehrzadi[g], Mohammad Taghi Rahimi[h], Zahra Mirabediny[a,b], Monireh Golpour[b,i], Mahbobeh Montazeri[a,b,d,*]

[a] Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Iran
[b] Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran
[c] Department of Parasitology, School of Medicine, Sari Branch, Islamic AZAD University, Sari, Iran
[d] Department of Parasitology and Mycology, Sari Medical School, Mazandaran University of Medical Sciences, Sari, Iran
[e] Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
[f] Department of Biochemistry, Biophysics and Genetic, Cellular and Molecular Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[g] Razi Drug Research Center, Iran University of Medical Sciences, Tehran, Iran
[h] School of Medicine, Shahroud University of Medical Sciences, Shahroud, Iran
[i] Molecular and Cell Biology Research Center, Mazandaran University of Medical Sciences, Sari, Iran

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ABSTRACT

Toxoplasmosis is a worldwide disease caused by the protozoan parasite *Toxoplasma gondii* (*T. gondii*), which is most commonly treated by pyrimethamine and sulfadiazine. However, this treatment presents several adverse side effects; Thus, new drugs with lower toxicities are urgently needed. In this study the anti-*T. gondii* activity of *A. vera* and *Eucalyptus* extracts were evaluated *in vitro* using a MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide) assay and *in vivo* by measuring the survival rates of mice infected with 2×10^3 tachyzoites of RH strain of *T. gondii* and then injected intraperitoneally by different concentrations of extracts for 4 days. Biochemical parameters such as Ferric Reducing Antioxidant Potential (FRAP) and malondialdehyde (MDA) assay were also evaluated. As results, in the *in vitro* assay, the IC_{50} values were 13.2, 24.7, 2.63 μg/ml, and the selectivity indexes were 3.3, 2.4, 3.03 for the *A. vera, Eucalyptus* and pyrimethamine, respectively. The mice treated with *Eucalyptus* showed a better survival rate than others ($P < 0.05$). The increased weight of liver and spleen, due to infection, was reduced by treatments. In FRAP assay *Eucalyptus* showed a better antioxidant activity than the other extracts. MDA levels in both liver and spleen were reduced by treatment. The results show that *A. Vera* and *Eucalyptus* possess anti-*T. gondii* activities *in vitro* and *in vivo*, in addition, *Eucalyptus* shows antioxidant activity with a higher survival rate. Therefore, *Eucalyptus* may be a useful candidate for treating *Toxoplasma* infection. Moreover, further studies are required to investigate the fractionations of this plant against *T. gondii*.

1. Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite of the Apicomplexa phylum that infects a wide range of hosts, including humans and other warm blooded animals (Faucher et al., 2012). There are three infective stages of *T. gondii*: a rapidly dividing invasive tachyzoite, a slowly dividing bradyzoite in tissue cysts, and an environmental stage, in which the sporozoites is protected inside an oocyst (Robert-Gangneux and Dardé, 2012). People typically become infected by three principal routes of transmission: food borne, animal to human (zoonotic) and mother-to-child (congenital), and rarely as post-solid organ transplant or blood transfusion (Scallan et al., 2011).

Approximately one third of humans have been infected with *T. gondii* (Demar et al., 2012). In immune competent hosts the acute infection is mild, self-limited and persisting in a latent form as tissue cysts (Mozzatto and Procianoy, 2003). However, the infection in immune compromised patients, represents serious problems for general health and it may cause retinochoroiditis and fatal encephalitis if not treated (Kim and Weiss, 2004). Additionally, *T. gondii* infection during pregnancy may cross the placental barrier, causing neurological damage to

* Corresponding author. Department of Parasitology and Mycology, Sari Medical School, Mazandaran University of Medical Sciences, PC 48168-95475, Sari, Iran.
E-mail address: m.montazeri2012@yahoo.com (M. Montazeri).

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the fetus and miscarriage (Mozzatto and Procianoy, 2003). There is no vaccine available to prevent human infection with this pathogen (Ahmadpour et al., 2017a, 2017b).

Currently, toxoplasmosis is being treated with a combination of sulfadiazine and pyrimethamine (PRY) (Wei et al., 2015). Unfortunately, the available treatments have significant toxicity, including suppression of the bone marrow and teratogenic effects in the first trimester of pregnancy (Değerli et al., 2003; Schmidt et al., 2006). Moreover, these therapies are effective against tachyzoites in the acute stage of the disease and have no effect over the bradyzoite form (Montazeri et al., 2017). Thus, development of new treatment options for toxoplasmosis with less toxic effects are extremely important (Montazeri et al., 2016). Natural herb extracts and medical plants are widely used as alternative treatment for various parasitic diseases and considered to be safe and to have low toxicity compared to synthetic drugs (Ebrahimzadeh et al., 2017).

Aloe vera (A. vera) is one of the Liliaceae family of which there are about more than 400 species and it grows wild in tropical climates around the world and it is also cultivated for agricultural and medical uses (Newall et al., 1996). This plant is one of the oldest medical herb that has been used to treat wounds and reduce fever (Ahlawat and Khatkar, 2011). Pharmacologically the properties of *A. vera* plants have been shown to be anti-bacterial, anti-inflammatory, anti-arthritic, anti-diabetic, anti-fungal, anti-cancer, wound healing and gastro protective (Jani et al., 2007; Newall et al., 1996; Yadav et al., 2015).

Eucalyptus, a large genus of Myrtaceae family, represented by 900 species and subspecies, is native to Australia and can be found all around the world. Native Australians used *Eucalyptus* leaves for wound healing and treating fungal infections (Gilles et al., 2010). According to previous studies, *Eucalyptus* has beneficial biological effects such as, anti-microbial, anti-hyperglycemic, anti-oxidant activities and anti-trichomonas activity (Mahdi et al., 2006; Takahashi et al., 2004; Youse et al., 2012). Thus, we were interested in exploring whether *Eucalyptus* and *A. vera* plants possess anti-*T. gondii* activity. The aim of this study was to evaluate the effects of *Eucalyptus* and *A. vera* methanol extracts on *T. gondii* infections *in vitro* and *in vivo*.

2. Materials and methods

2.1. Plant material

The fresh leaves of *A. vera* and *Eucalyptus* were purchased from herbal drug stores in Mazandaran province and were confirmed by Dr. B. Eslami, Assistant Professor of Plant Systematic and Ecology (Department of Biology, Islamic Azad University of Qhaemshahr, Iran). The plant serial numbers for *A. vera* are as follows: 34–93 and for *Eucalyptus*: 18–93. Fresh leaves were cleaned with water and only sound leaves were dried at room temperature and then powdered. Each herb was extracted by the percolation method, with methanol at room temperature. The air-dried powdered leaves were extracted with 97% methanol for 3 days at 50 °C temperature and filtrated with sterile cotton gauze. The filtrate was evaporated to dryness under reduced pressure with rotary evaporator (Heidolph-4000, made in Germany), and then lyophilized using freeze Dry Vacuum System. The yields of the extracts based on their dry weights (100 g) were 17.1% for *Eucalyptus* and 10% for *A. vera*.

2.2. T. gondii strain

The RH strain of *T. gondii* was provided by the Toxoplasmosis Research Center (TRC) in Mazandaran University of Medical Sciences, Sari, Iran. Tachyzoites were maintained by intraperitoneal (IP) passages in female Balb/c mice (8-10 week-old, 20–25 g weight), 3–4 day after IP injection with 1×10^5 of the parasite. All mice were housed in cages under standard laboratory conditions including an average temperature (20–25 °C), humidity (60 ± 10%), light (12 h per day), given drinking water, and regular diet in the animal center of Mazandaran University of Medical Sciences, Sari, Iran.

The tachyzoites were collected from the peritoneal exudates of infected mice, washed three times, and then got diluted with phosphate -buffered saline (PBS) PH: 7.4, containing 100 IU/ml of penicillin and 100 µg/mL of streptomycin (Montazeri et al., 2015). The animal protocols used in this research were approved by the Mazandaran University of Medical Sciences Ethics Committee (MUMSEC) (Permit number 492).

2.3. Cytotoxicity tests

Vero cells, kidney fibroblast from African green monkey (ATCC No. CCL-81), were used for *in vitro* assays; cultured in RPMI-1640, supplemented with 10% inactivated fetal bovine serum (FBS), 100 µg/ml penicillin and streptomycin and maintained at 37 °C in 5% CO2. All agents were dissolved in complete culture medium RPMI 1640 with less than 1% dimethyl sulfoxide (DMSO). Vero cells were plated in 96-well plates (cell suspensions 2×10^4 cell/ml in complete culture medium RPMI 1640) and incubated at 37 °C in 5% CO_2 for 24 h. Next the cells were exposed to the *A. vera*, *Eucalyptus*, and PRY at final concentrations of (5- 10- 25- 50- 100- 200- 400–600 µg/ml) and RPMI 1640 was used as a control. After 24 h, the cell viability was measured by adding MTT solution (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to the cultures (Choi et al., 2013). The absorbance of the supernatant was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Synergy H1/USA). Then the 50% cytotoxic concentrations (CC_{50s}) were calculated using the Graph Pad Prism 6.0 software (Graph Pad Software, Inc., San Diego, USA).

2.4. Effects of A. vera and Eucalyptus extracts on intracellular T. gondii in vitro

Vero cells were cultured in 96-well plates (2×10^4 cell/well) in complete culture medium RPMI 1640 for 24 h at 37 °C and 5% CO2. Next, the cells were infected with the RH strain of *T. gondii* tachyzoites (parasite: cell ratio = 10:1). After 24 h the medium was changed in order to remove extracellular parasites and then incubated with different concentrations (5–600 µg/ml) of *A. vera* and *Eucalyptus* extracts. PRY and RPMI 1640 culture medium were used as positive and negative control, respectively. After 24 h, MTT solution (5 mg/ml) was added to the culture wells and then incubated for 4 h at 37 °C in 5% CO_2 atmosphere. Finally, 200 µg/well DMSO were added to all plates. After 15 min, the optical absorbance was measured at the 570 nm wavelength. The growth inhibition concentration was calculated and the mean 50% growth inhibition concentration (IC_{50}) was estimated from the dose-response curves of *A. vera* and *Eucalyptus's* different concentrations by using the Graph Pad Prism 6.0 software. In addition, selectivity index (SIs) of the drugs were calculated using the IC_{50} and the host-cell cytotoxicity profiles ($SI = CC_{50}/IC_{50}$).

2.5. Effects of A. vera and Eucalyptus extracts on T. gondii infections in vivo

Female Balb/c mice, were divided into 7 groups (n = 6). One group did not get inoculated with any tachyzoites (uninfected control). Six groups were IP inoculated with *T. gondii* (RH strain, 2×10^3 tachyzoites per mouse); after 4 h post-infection, mice got injected with different

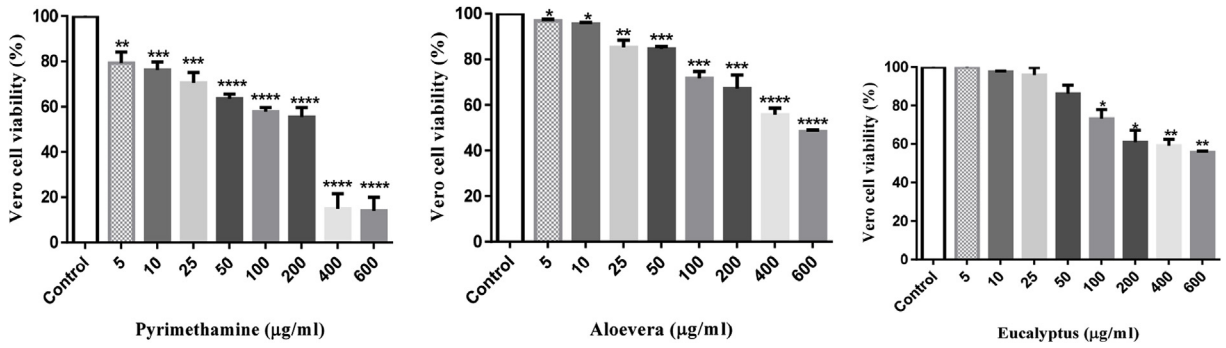

Fig. 1. Cellular viability in different concentration of *A. vera*, *Eucalyptus* and PRY in Vero cell cultures. Values are expressed as mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with control.

agents. The injections were PBS for the infected control group, PRY for the positive control group, *A. vera* extract 50 and 100 mg/kg/day (groups 4 and 5 respectively) and *Eucalyptus* extract 100 and 200 mg/kg/day (groups 6 and 7 respectively). Injections were done once a day for 4 days. Four days post infection, mice body weights were measured and then blood samples were collected from heart. Afterwards, animals were sacrificed and their spleens and livers were weighted and stored for further tests analysis at −20 °C.

2.6. Survival rates

Six groups consist of 6 mice in each were chosen in this parts of study. All groups were IP injected with 2×10^3 tachyzoites of *T. gondii*. After 4 h, the infected control group were treated with PBS, the positive control group was treated with PRY (50 mg/kg/day), 4 other groups treated with *A. vera* (50 or 100 mg/kg/day) or *Eucalyptus* (50 or 100 mg/kg/day) for 4 days. Mice were observed daily until the last mouse was found dead, and the number of dead mice was noted. At the end survival rate in the mice was calculated.

2.7. Ferric Reducing Antioxidant Potential (FRAP) assay

Anti-oxidant capacity of plasma was evaluated using FRAP assay (Dudonné et al., 2009; Luqman et al., 2012). FRAP Assay is based on the reduction of colorless ferric complex (Fe^{3+}-TPTZ) to a blue-colored ferrous complex (Fe^{2+}-TPTZ). The reducing properties associated with the presence of compounds exert their action by breaking the free radical chain through donating hydrogen atom (Duh et al., 1999; Gordon, 1990). Blood samples were collected from heart with the heparinized syringes. Then the heparinized blood samples were centrifuged (4000 rpm) to separate the plasma, afterwards 50 µl of plasma was added to 1.5 ml of FRAP solution (mixture of HCl (40 mM), acetate buffer (300 mM, PH = 3.6), $FeSO_4$ (1 mM) $FeCL_3$ (20 mM), and TPTZ (10 mM)) in a micro tube and the mixture was incubated for 10 min in 37 °C, then absorbance of all samples were measured at 593 nm using an uv-1800 spectrophotometer (SHIMADZU/China).

2.8. Measurement of liver and spleen weights and MDA levels

Livers and spleens of all groups were weighed in order to compare them with uninfected control group and then MDA was measured using the thiobarbituric acid reaction. Homogenate liver and spleen was added to a mixture of SDS (8.1%, 0.2 ml), Thiobarbituric acid (8%, 1.5 ml), acetic acid (PH 3.5, 1.5), and water (0.6 ml), it was incubated for 1 h, and then cooled with ice. After an addition of n-butanol (1 ml), the mixture was centrifuged at 4000 rpm for 10 min and the absorbance of the organic layer was measured at 532 nm. The values are expressed in nM/gm of tissue (Ohkawa et al., 1979).

2.9. Statistical analysis

Statistical analysis were performed on all data using Graph Pad Prism 6.0 software. Differences between test and control groups were analyzed by analysis of variance (ANOVA) and the Newman-Keulsmultiple-comparison test. Also, the Kaplan–Meier curve was used to show the survival times, and differences were compared using the log-rank test. Statistical significance was assumed as $P < 0.05$.

3. Results

3.1. Cellular viability in different concentration of A. vera, Eucalyptus and PRY

To analyze the toxicity of PRY and extracts on Vero cells *in vitro*, we investigated cell viability using the MTT assay. The treatment with *Eucalyptus* at concentrations of 5–50 µg/ml did not significantly affected cell viability compared to cells treated with medium alone. Vero cells treated with *A. vera* and *Eucalyptus* at used concentrations (ranging from 5 to 600 µg/ml) showed higher cell viabilities than PRY (Fig. 1).

3.2. Effects of the A. vera and Eucalyptus extracts and PRY on T. gondii in vitro

To analyze the anti-*Toxoplasma* effects of each extracts, different concentrations of the *A. vera*, *Eucalyptus* and PRY were examined. *A. vera* showed anti-*Toxoplasma* activity with a selectivity index (SI) at 3.3. In addition, PRY activity was characterized by an IC_{50} of 2.63 µg/ml and SI of 3.03 (Table 1).

Table 1
In vitro anti-*T. gondii* activity of *A. vera* and *Eucalyptus*.

Extract/drug	CC_{50}[a](µg/ml)	IC_{50}[b] (µ/ml)	SI[c]
A. vera	43.2	13.2	3.3
Eucalyptus	58.7	24.7	2.4
PRY	7.99	2.63	3.03

Results are presented as the mean IC_{50} and CC_{50} values obtained from three independent experiments.

[a] Concentration required to reduce Vero cell growth by 50% (µg).

[b] Concentration required to inhibit *T. gondii*-induced cytopathic effect by 50% (µg).

[c] Selectivity index = CC_{50}/IC_{50}.

3.3. Survival rate

One of the mice of the infected control group died in the sixth day of experiment, and the other (n = 5) died on the seventh day post infection. Mice in the groups treated with extracts showed higher statistically significant survival rates than the mice in the infected control group ($P < 0.05$). However, the best survival rate was observed in mice receiving *Eucalyptus* 100 mg/kg compared to the other extracts (Fig. 2).

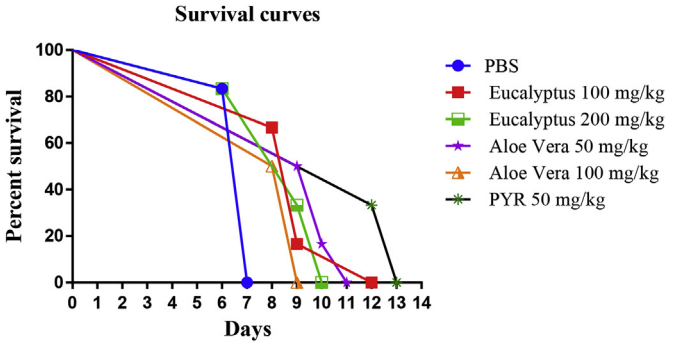

Fig. 2. The survival curves of mice following acute toxoplasmosis. Balb/c mice (n = 6) infected with 1×10^3 tachyzoites of the *T. gondii* RH strain were treated with *Eucalyptus* (100, 200 mg/kg/day), *A. versa* (50, 100 mg/kg/day) and pyrimethamine (50 mg/kg/day) (as the positive control) and PBS (as the infected control) for 4 days via the intraperitoneal route.

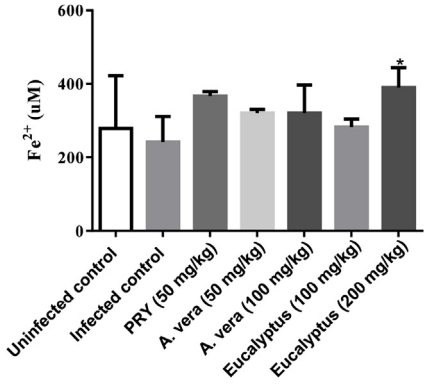

Fig. 3. FRAP values represent as equivalent mol of Fe^{2+}/gram sample. Results are expressed as mean ± SD obtained from six plasma samples in each group. *$P < 0.05$ compared with infected control.

3.4. FRAP test

Heparinized blood samples were centrifuged (4000 rpm) to separate the plasma, then 50 µl of plasma was added to 1.5 ml of FRAP reagent. The reaction mixture was incubated in a water bath for 30 min at 37 °C; then, the absorbance of the samples was measured at 593 nm. The difference between absorbance of samples and the absorbance of blank was calculated and used to calculate FRAP value (Fig. 3). The absorbance of *Eucalyptus* (200 mg/kg group) was higher than the other extracts. There was no significant difference among test groups and uninfected control group.

3.5. Effect of A. vera and Eucalyptus on liver and spleen weights

In order to evaluate the effect of *A. vera* and *Eucalyptus* on liver and spleen, the liver and spleen index were calculated as liver weight/body weight and spleen weight/body weight. The liver and spleen index in the infected control group increased compared to the uninfected control group. The liver index was decreased in the *A. vera* and *Eucalyptus* (100 mg/kg/day) groups (Fig. 4). Treatment with *A. vera*, *Eucalyptus* and PRY reduced the increment in the spleen index which was caused by the *T. gondii* infection (Fig. 4).

3.6. MDA level

The MDA level of liver and spleen was significantly elevated in the infected control group compared to the uninfected control group. It was shown that all treatment groups had statistically significant reductions of MDA level compared to infected control mice (Fig. 5).

4. Discussion

Current standard therapies against toxoplasmosis are limited, and drugs have severe side effects and low efficacies. In this respect, the search for alternative compounds with novel mechanisms of action is urgently needed (Montazeri et al., 2017). Natural compounds and traditional herbal medicine have high availability and lower side effects compared to current chemical agents (Ebrahimzadeh et al., 2017). Therefore, the aim of the present study was to determine the activity of *A. vera* and *Eucalyptus* during the experimental infection with *T. gondii*, which had not been previously reported.

According to the *in vitro* results, *A. vera* and *Eucalyptus* showed higher CC_{50} than PRY in Vero cells. Interestingly, *Eucalyptus* at concentrations of 5–50 µg/ml did not significantly reduce the cellular viability in Vero cells. Also, the extracts, especially *A. vera*, showed an anti-*T. gondii* activity against infected Vero cells. Moreover, the SI of the methanolic extract of *A. vera* was higher than standard treatment.

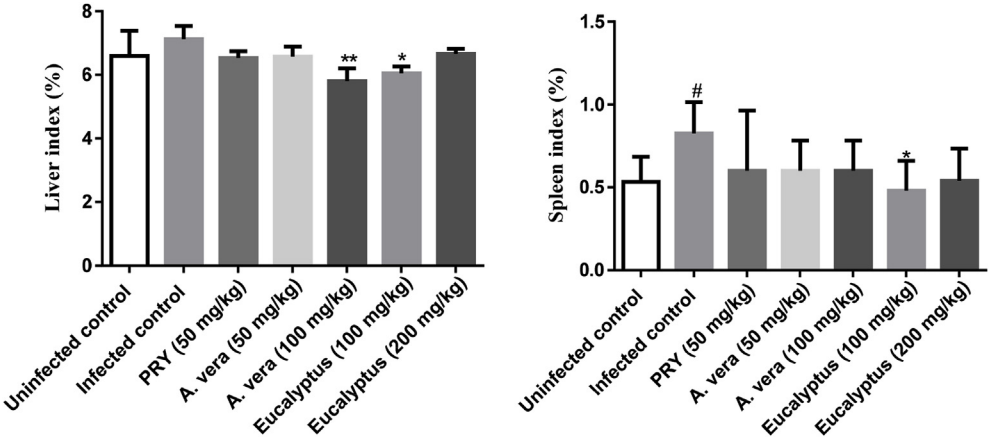

Fig. 4. Effects of *A. vera* and *Eucalyptus* on weight change of liver and spleen in *T. gondii* infected Balb/c mice. Liver index: Liver weight/body weight × 100; Spleen index: Spleen weight/body weight × 100; Values are expressed as mean ± S.D. *$P < 0.05$, **$P < 0.01$ compared with infected control. #$P < 0.05$ compared with uninfected control.

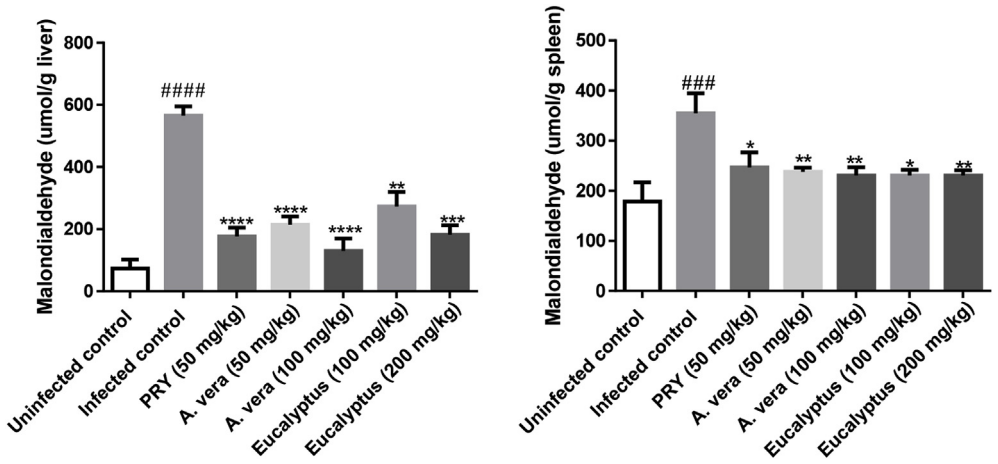

Fig. 5. Effect of *A. vera* and *Eucalyptus* on MDA levels in *T. gondii*-infected livers and spleens. Values are expressed as mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared with infected control. $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared with uninfected control.

Similarly, Iqbal et al. observed that methanolic extract of *A. vera* leaf had a significant antipromatigote activity against *Leishmania tropica in vitro* conditions (Iqbal, 2012).

The effect of *A. vera* and *Eucalyptus* on the survival rates of Balb/c mice infected with tachyzoites of RH strain of *T. gondii* was also examined. We used only two doses of the extracts in the *in vivo* experiment, based on the efficacy and toxicity. *Eucalyptus* and *A. vera* increased the survival rate of the mice compared to the untreated infected control. Interestingly, the mice treated with *Eucalyptus* (100 mg/kg/day) exhibited a better survival rate than those treated with the other extracts. Given that *Eucalyptus* 100 mg/kg/day and *A. vera* 50 mg/kg/day showed higher viability on Vero cells in comparison to *Eucalyptus* 200 mg/kg/day and *A. vera* 100 mg/kg/day, these doses achieved better results in mice survival rates *in vivo*.

Ebrahimzadeh et al. showed the effects of different herbal drugs against *T. gondii* infection *in vivo* on RH strain. In this study treatment of *T. gondii* infection with *Feijoa sellowiana, Quercus castaneifolia,* and *Allium paradoxum* methanol extracts increased survival rates compared to untreated group, and *F. sellowiana* leaves achieved better survival time in the mice compared to other extracts (Ebrahimzadeh et al., 2017).

Previous studies revealed that *T. gondii* infection can lead to oxidative stress (Elsheikha et al., 2009). Xu et al. showed that antioxidants do have potential as a therapeutic regimen for treatment of *T. gondii*-related diseases (Xu et al., 2012). In this study, the mice treated with the extracts, showed an increase in the antioxidant activity, especially *Eucalyptus* (200 mg/kg). Our findings suggest that mechanism of action of *A. vera* and *Eucalyptus* could possess antioxidant effect during *T. gondii* infection.

Eucalyptus extracts contain polyphenols, terpenoid and cineol that had previously displayed remarkable antioxidant activity (El-Moein et al., 2012). Angelo Luis et al. showed eucalyptol, the principal compounds of the essential oils of Eucalyptus species, exhibit antioxidant activity due to the presence of phenolic compounds (Luís et al., 2017).

Saritha et al. indicated the efficacy of *A. vera* methanol extract as a source of natural antioxidants *in vitro* which might have application towards reducing lipid peroxidation/oxidative stress with consequent health benefits (Saritha, 2010).

The liver is a major target organ in metabolism system of organisms (Yang et al., 2010). Choi et al. showed that the relative weight of liver and spleen significantly increases after infection with *T. gondii* (Choi et al., 2014). In this study the liver/body weight ratio as a marker for liver affection and toxicity (as well as spleen/body weight ratio) in *T. gondii* infected mice treated with extracts reduced slightly compare to infected control, and there is no significant difference among tests and uninfected control group. Zhang et al. reported that the mice treated with sophora leguminous plants, Martine and Oxymartine, achieved better results in viscera index than spiramycin (Zhang et al., 2016).

Present study revealed that MDA, the main product of lipid peroxidation, in the liver and spleen of mice infected with *T. gondii* was significantly increased compared to uninfected control. Treatment with extracts resulted in a significant reduction in MDA level. These results reflect the oxidative stress induced by *T. gondii* infection which is in accordance with the findings of Zhang et al. who reported a decrease in MDA level in the serum of mice treated with Martine. Findings of this study reflects the potent antioxidant effect of *Eucalyptus* and *A. vera*. Similarly, Mady et al., reported that treatment of mice with *Nigella sativa* oil significantly reduced the serum lipid peroxidation marker, MDA (Mady et al., 2016).

In conclusion, the results of this study demonstrated for the first time that *A. vera* and *Eucalyptus* show anti-*T. gondii* activities *in vivo* and *in vitro*. Although survival time in mice treated with extracts was relatively lower than the available market drug (PYR), *A. vera* and *Eucalyptus* could be a novel drug candidate. Especially, *Eucalyptus* showed antioxidant activity with a higher survival rate *in vivo*. Additional studies are required to investigate the fractionations of this plant against *T. gondii* parasite.

Conflicts of interest

There is no conflict of interests.

Author contributions

AD, MSH, SS, MZ and MAE designed the study. BA, MM, MTR, ZM, MAE, MG performed the experiments. MM and MZ analyzed the data. BA and MM drafted the manuscript. SM critically revised the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2018.07.010.

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