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Original Article

Anti-*Toxoplasma* Effect of Hydroalchohlic Extract of *Terminalia chebula* Retz in Cell Culture and Murine Model

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Abstract

Background: We examined anti-*Toxoplasma* effect of hydroalcoholic extract of *Terminalia chebula* Retz (*T. chebula*) in cell culture and murine model.

Methods: The study was conducted in Shahrekord University of Medical Sciences, Iran in 2017. Half maximal effective (concentration (EC50) of *T. chebula* extract and pyrimethamine was determined in infected Hela cells by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) method. In the animal model, BALB/c mice were injected with tachyzoites (10^4) of *T.* RH strain intraperitoneally. 24h after the injection, the test groups were orally treated with 100, 200, 400 and 800 mg/kg of *T. chebula* extract for 7 days. The survival rate of the mice was determined and blood samples were collected to determine the amount of serum Malondialdehyde (MDA) and antioxidant capacity. Then peritoneal fluid of the mice was collected to count the number of tachyzoites and after necropsy, the pathologic changes, including the weight of liver, spleen and kidneys were investigated. The analysis of data was accomplished using SPSS.

Results: EC_{50} values were 94.7µg/mL and 290.50µg/mL for *T. chebula* and pyrimethamine respectively. In the animal model, the extract of *T. chebula* in concentration of 100 mg/kg showed the same anti-*Toxoplasma* effect as pyrimethamine. This concentration of the extract decreased number of intraperitoneal tachyzoites and increased the survival rate of the mice. This extract reduced the levels of serum MDA and tissue inflammation and increased serum antioxidant capacity.

Conclusion: Regarding the positive effect of extract, after more clinical trials in the animal model and standardization of the extract, it can be used as an alternative or complementary therapy for toxoplasmosis.

Introduction

Toxoplasma infection caused by the obligate intracellular protozoan parasite, *T. gondii* is one of the most prevalent zoonosis with a wide world distribution (1). The infection is commonly transmitted to humans through consumption of raw or undercooked meats containing toxoplasma tissue cysts or water and foods contaminated with oocysts of the parasite (2-4).

The acute *T. gondii* infection is generally benign and self-limited in immunocompetent individuals (5, 6), but it can adversely affect the immunodeficient patients with severe and even fatal outcomes such as encephalitis, pneumonitis and myocarditis (7). Moreover, during pregnancy toxoplasmosis may induce an acute and severe infection in growing fetus such as hydrocephaly, microcephaly, mental retardation and chorioretinitis (8, 9).

At present, a combination of pyrimethamine and trisulfopiramidines is used as the standard regimen for treatment of acute toxoplasmosis (6). However, the prescription of these drugs is not allowed during first trimester of pregnancy and it may also induce side effects, including bone-marrow toxicity, crystalluria and sometimes acute ureteric obstruction in patients (10-12). For these reasons, some studies have long been carried out to find and replace herbal remedies for treatment of different microbial infections, including toxoplasmosis (13). In this way, the antibacterial, antifungal, antiviral and antiparasitic (antiamebic and antimalaria), anticanceogenic and antidiabetic effects of *T. chebula*, a plant of Combretaceae family have been confirmed in some studies (14-23).

We aimed to investigate the anti-*Toxoplasma* effect of hydroalcoholic extract of *Terminalia chebula* Retz in cell culture and murine model.

Materials and Methods

Extraction of Terminalia chebula Retz

The plants were purchased from medicinal plant stores and identified as the plants of interest by botanists; one voucher specimen (no. 27) was deposited for *T. chebula* at the Herbarium of Shahrekord University of Medical Sciences Chaharmahal and Bakhtiari Province in 2017. We used about 200 gr of fruit and the maceration method for extraction of the plant. Briefly, the plant was powdered and soaked in 70% ethanol for 72 hours. The obtained extract was concentrated by rotary evaporator at 38 °C. For complete drying, the extract was placed in a 40 °C incubator and after that, dried extract was stored at -20 °C (24).

Preparation of Toxoplasma gondii tachyzoites

The standard RH strain of *T. gondii* was provided by Department Of Parasitology, the Isfahan School of Medicine. For parasite proliferation, about one million tachyzoites were injected to 3-4 mice, intraperitoneally. Forty-eight to seventy-two hours after the injection, the peritoneal fluid of the mice was aspirated and centrifuged at 4,000 rpm for 7 minutes. Finally, the number of tachyzoites was counted by using hemocytometer slide under invert microscope (25).

Cell culture

Hela cells were cultured in 25 mL flasks containing RPMI1640 (Gibco) medium enriched with 10% Fetal calf serum (FCS Gibco) were incubated at 37 °C and 5% CO_2. To transfer cells to 96-well plates, the cells were first subjected to trypsin and then washed twice with phosphate-buffered saline (pH 7.2 - 7.4). Finally, the number of viable cells in suspension was determined by Trypan blue staining and Neubauer hemocytometer (26).

In vitro anti-Toxoplasma assay

After mass cultivating of Hela cells, to determine Cytotoxic Concentration (CC_{50}) on

Hela cells, by using RPMI 1640 medium a dilution of cells were prepared so that there were 10^4 cells per 100µL. In the next step, the cells were treated with *T. chebula* extract for 24 h (31.5 to 1500 µg/mL), the extract was easily dissolved and sterilized with a 0.2 µm filter. After the incubation time, MTT test was carried out. To determine EC_{50} of the extract on infected Hela cells with tachyzoites, by using RPMI 1640 medium a dilution of cells was prepared so that there were $6x10^4$ cells per 100µL. The plates were incubated at 37 °C for 24 hours. Then 100µL of the suspension contained $3x10^5$ tachyzoites were added to each well (the ratio of tachyzoites to cells: 5/1) and the plate was incubated at 37 °C for 6 hours. For elimination of extracellular tachyzoites, the content of wells was then washed two times with free calf serum RPMI1640 medium. The plates were incubated for 18 h at 37 °C and subsequently were treated with *T. chebula* extract (3 .5 to 250µg/mL) and pyrimethamine (12.5 to 550 µg/mL)(5-(4-Chlorophenyl)-6-ethyl-2,4-pyrimidinediamine, Sigma-Aldrich). After 24 hours, the MTT test was accomplished (22, 27, 28).

In vivo experiments

For this purpose, seventy BALB/c mice were randomly divided into 7 groups so that each group consisted of 10 mice. In the first group (control group), 0.5 mL sterile normal saline was injected intraperitoneally. The mice of the second group (Toxoplasmosis control= Untreated group), third group (positive control) and four other therapeutic groups received $1x10^4$ *toxoplasma* tachyzoites, intraperitoneally. Twenty-four hours after tachyzoites injection, the mice of the third group received daily 12.5 mg/kg of pyrimethamine for 7 d by oral gavage (13, 29). Simultaneously, the mice of four other therapeutic groups received 100, 200, 400 and 800 mg/kg of *T. chebula* extract for 7 d orally. After the injection of *toxoplasma* tachyzoites, signs, symptoms and mortality rates of mice were evaluated daily and finally, the survival rate was determined. After the end of the course of treatment, in the eighth day, blood samples mice were collected to determine the antioxidant capacity and the amount of MDA, as a marker for oxidative stress. The peritoneal fluid of mice received the parasite suspension was also collected to determine the number of tachyzoites contained. In the final stage of experiment, after anesthetizing, the animals were killed and the kidneys, liver and spleen were collected for weight variation. The mean survival rate of treated mice was also evaluated (30-32).

This study has received the code of ethics IR.SKUMS.REC.1395.196 from the Research and Technology Deputy of Shahrekord University of Medical Sciences.

Measurement of Serum Malondialdehyde

The measurement of serum MDA was carried out according to Uchiyama-Mihara method. Briefly, 1 mL of 0.06% Thiobarbituric acid, 3 mL of 1% phosphoric acid and 0.5 mL of sera from the mice were added to a test tube and the mixture was incubated in boiling Bain-marie for 45 minutes. After cooling of the mixture, 4 mL of n-buthanol was added to test tube, the amount of serum MDA was measured using the Unico spectrophotometer at 532nm wavelength, and the results were recorded as nmol/L (33).

Determination of serum antioxidant activity

To determine serum antioxidant capacity the ferric reducing/antioxidant power (FRAP) method was used. Briefly, 1.5 mL of fresh FRAP reagent was added to a test tube containing 25µL serum and the mixture was incubated at 37 °C for 10 minutes. In this method, the complex formed between Fe^{+2} and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) causes a blue color at 593 nm wavelength. In this method, $FeSo_4.7H_2O$ was used as standard in concentration range of 100-1000µmol (34, 35).

Data analysis

The data were summarized for each group using percentage for qualitative, means ± standard deviation for quantitative and normally distributed, or medians with interquartile range (IQR) for quantitative and non-normally distributed variables. Normality distribution was assessed with the Kolmogorov test. Differences between groups were analyzed using ANOVA with Tukey Post Hoc test, Kruskal-Wallis followed by Dunn's Post Hoc test. EC50 was determined using probit regression. The survival rate was compared using Log-rank test. Statistical significance was defined as $P<0.05$ in all tests and analysis was performed by SPSS (ver. 23, Chicago, IL, USA). Graphs were drawn using GraphPad Prism ver. 7.

Results

In vitro results

EC_{50} of *T. chebula* extract and pyrimethamine on Hela cells and *Toxoplasma* tachyzoites was determined using MTT method and their values were calculated by dose-response curve and regression analysis (Table 1). The study showed that in vitro, *T. chebula* extract had a significant effect more than pyrimethamine on *T. gondii* tachyzoites.

Table 1: CC_{50} of *T. chebula* Retz in Hela Cell Culture and EC_{50} in contaminated Hela cells with *T.gondii* tachyzoites

Extract and drug	*CC_{50}(µg/mL) (95% CI) Hela cell*	*EC_{50}(µg/mL) (95% CI) Contaminated Hela cell with T. gondii RH*	*Selectivity[a]*
Hydro-alcoholic extract of *Terminalia chebula* Retz	508.5 (448.7 – 581.44)	94.7 (51 – 366)	5.36
Pyrimethamine	743.6 (694.4 - 797.8)	290.5 (226.6 – 381.5)	2.5

Values are expressed as mean and 95% confidence interval and probit regression analysis
a Ratio of the EC50 value for HeLa cells to the EC50 value for *T. gondii* RH strain

In vivo results

The Kruskal–Wallis test showed that the number of *Toxoplasma* tachyzoites in the peritoneal fluid of test groups treated with different doses of *T. chebula* extract was significantly decreased compared with positive control and toxoplasmosis control group (Table 2). The number of tachyzoites in peritoneal fluid of the mice treated with 100 mg/kg and 200 mg/kg doses of the extract was significantly decreased compared with positive control and toxoplasmosis control group (P=0.001). However, there was no statistically significant decrease of the parasites in peritoneal fluid of mice treated with doses of 400 mg/kg and 800 mg/kg of the extract compared with toxoplasmosis control group. Therefore, according to data analysis in test groups that received different doses of *T. chebula* extract, the anti-toxoplasmic effects of the extract do not appear to be dose-dependent.

Results of mean survival rate

In this section of study, the mortality rate of the subjects was determined. According to Log-rank test, the survival rate of mice in different groups was compared and analyzed (Table 3). The mortality rate of mice treated with 100 mg/kg of *T. chebula* extract was similar to positive control group (40%) and there was a significant difference in survival of mice in investigated groups (P=0.001).

Table 2: Number of tachyzoites in peritoneal fluid collected from *T. gondii* infected animals treated with hydroalcoholic extract *T. chebula* after 7 days

Variable *Group*	*Median (Interquartile Range (IQR)) (×10³)*
Control	0 (0 -0)
Toxoplasmosis control	25280 (24000-26400)
Positive control	13333 (10825-14400)
The group receiving the dose of 100 mg/kg extract	11616 (11000-12075)
The group receiving the dose of 200 mg/kg extract	12960 (12000- 13900)
The group receiving the dose of 400 mg/kg extract	16500 (16500-16500)
The group receiving the dose of 800 mg/kg extract	17300 (16800-17300)

Table 3: Survival rate of animals treated with hydroalcoholic extract of *T. chebula* after 7 days

Group	*Percentage of death in group*	*Mean survival rate/day*	*Standard Error*
Toxoplasmosis control	100	3.7	0.26
Positive control	40	7	0.447
The group receiving the dose of 100 mg/kg extract	40	7	0.253
The group receiving the dose of 200 mg/kg extract	50	6.9	0.386
The group receiving the dose of 400 mg/kg extract	90	5.8	0.395
The group receiving the dose of 800 mg/kg extract	80	5.6	0.514

Results of Serum Malondialdehyde levels

MDA levels in mice treated with 100 mg/kg of *T. chebula* extract were the lowest (9.87µmol), compared with toxoplasmosis control group and positive control group (Fig. 1). The analysis of variance test (ANOVA) indicated that there was a significant difference between the groups. The Post Hoc Tukey test showed that the serum levels of MDA in mice treated with 100 mg/kg and 200 mg/kg of the extract were significantly decreased compared with toxoplasmosis control group and positive control group ($P< 0.001$).

Serum antioxidant capacity results

The highest serum antioxidant capacity (1146.33µmol) between test groups was found in mice treated with 100 mg/kg of *T. chebula* extract, compared with toxoplasmosis control group and positive control group (Fig. 2). The analysis of variance test indicated that there was a significant difference between the groups. The Post Hoc Tukey test also showed that the serum antioxidant power in mice treated with 100 mg/kg and 200 mg/kg of the extract was significantly increased compared with toxoplasmosis control group and positive control group ($P<0.001$).

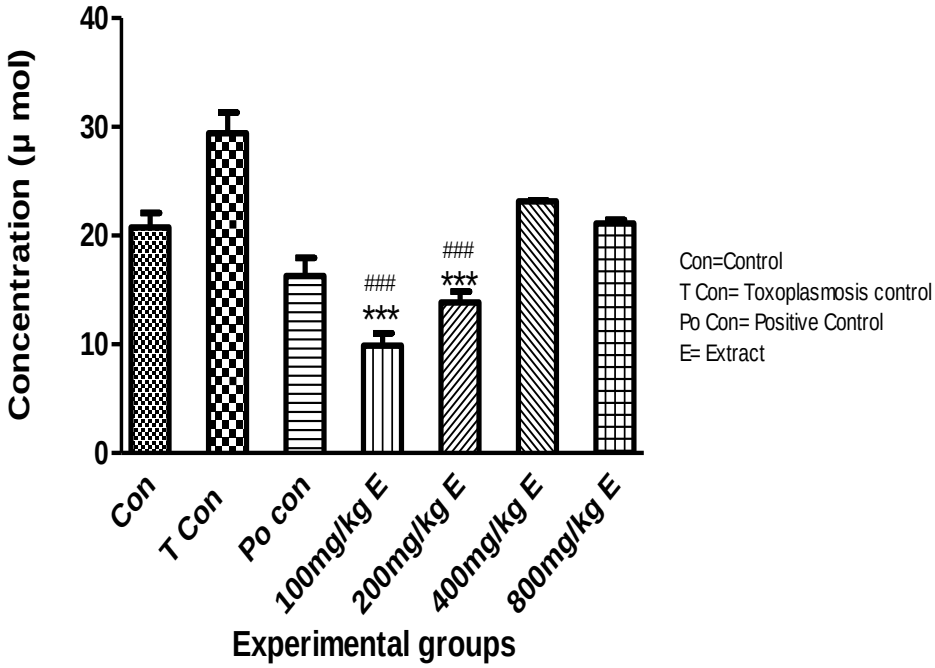

Fig. 1: Comparison of serum Malondialdehyde levels in the study groups
***P<0.001 compared with control and ### P<0.001 compared with positive control

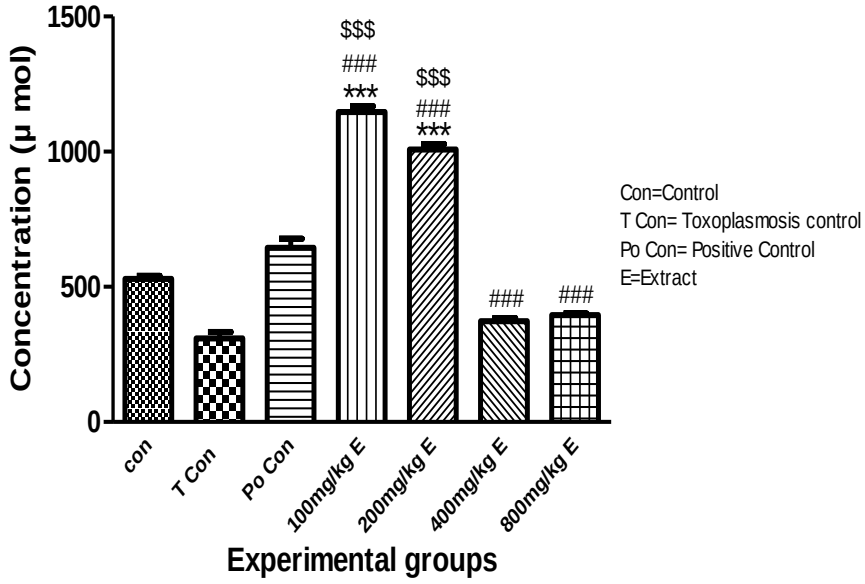

Fig. 2: Comparison of serum antioxidant capacity in experimental groups
*** P<0.001 compared with control, $$$ P<0.001 compared with toxoplasmosis control and ### P<0.001 compared with positive control

Organs weight changes

The Kruskal–Wallis test indicated that there was a significant difference between the weight of kidneys, liver and spleen of mice studied (Table 4). The Dunn test showed a significant decrease in kidney and spleen weight between the mice treated with 100 mg/kg and 200 mg/kg of the extract and toxoplasmosis control group and positive control group (P<0.05). There was also a significant decrease in the weight of liver between the mice treated with 100 mg/kg of the extract with toxoplasmosis control group (P=0.001).

Table 4: Weight changes of organs after treatment with hydroalcoholic extract of *T. chebula* in animal model Kruskal-Wallis test

Variable Group	Kidney (g)	Liver (g)	Spleen (g)
Control	0.269 (0.228 – 0.286)	1.550(1.315- 1.797)	0.390 (0.358 – 0.420)
Toxoplasmosis control	0.302 (0.288 -0.311)	2.700(2.590- 2.815)	0.459 (0.416- 0.590)
Positive control	0.292 (0.286 – 0.325)	1.750 (1.687- 2.012)	0.278 (0.269 – 0.282)
The group receiving the dose of 100 mg/kg extract	0.270 (0.251 – 0.301)	1.590(1.485- 1.685)	0.234 (0.190 – 0.336)
The group receiving the dose of 200 mg/kg extract	0.295 (0.247 – 0.303)	2.080 (1.965– 2.195)	0.255 (0.232 – 0.278)
The group receiving the dose of 400 mg/kg extract	0.352 (0.352- 0.352)	2.396 (2.396– 2.396)	.380 (0.380 – 0.380)
The group receiving the dose of 800 mg/kg extract	0.351 (0.302-0.351)	2.315 (2.300-2.315)	0.203 (0.202-0.203)

Data were presented as Median (Interquartile Range (IQR))

Discussion

Currently, the selective drugs for treatment of most cases of acute toxoplasmosis are a combination of pyrimethamine and sulfadiazine. The use of these drugs may cause a wide range of side effects such as, osteoporosis, teratogenic effects, crystalluria, hematuria and hypersensitivity reactions (36). Therefore, considering these risks and with regard to antimicrobial effects of some medicinal plants including, *T. chebula.*

This study was carried out to investigate and compare the anti-*Toxoplasm*a activity of *T. chebula* extract on *T. gondii* tachyzoites in cell culture and animal models with pyrimethamine. One of the most efficient methods to investigate and evaluate the effect of anti-*T. gondii* drugs are laboratory studies and animal models (37). In this study, EC50 of *T. chebula* extract was determined on growth inhibition of RH strain of *T. gondii* tachyzoites in Hela cell line. The inhibitory effect of *T. chebula* extract with selectivity index of 5.36 is much higher than that of perymethamine with selectivity index of 2.5. The selective index is a criterion for evaluating a compound's eligibility for being nominated as a drug candidate. Among 15 medicinal plants, the methanolic extracts of Ginger and Sophora with selective index of 10.1 and 4.6 had higher anti-*Toxoplasma* activity on Hela cultivated RH strain of *T. gondii* compared with pyriethamine with selective index of 2.1 (25).

We proposed that *T. chebula*, like many other medicinal herbs, has a potent anti-toxoplasmic effect with a higher selective index than pyrimethamine. Therefore, in this study, the anti-*Toxoplasma* effect of *T. chebula* extract was investigated in animal model. The LD50 of this extract was 2754.436 mg/kg (38). For the animal phase of study, dosages of 100, 200, 400, and 800 mg/kg of this extract were used. The

highest anti-toxoplasma effect of this drug was in the dosages of 100 and 200 mg/kg. In these dosages, *T. chebula* extract, similar to pyrimethamine, caused a significant decrease in growth rate of toxoplasma tachyzoites and increase the survival rate of the animals, unlike the treated group with the extract of *T. chebula*, all mice in the toxoplasmosis control group were died by fourth day. As expected, in the murine model, pyrimethamine in dosage of 12.5 mg/kg caused growth inhibition of *T. gondii* tachyzoites and survival of infected mice. This effect of pyrimethamine has been related to electron inhibition in mitochondria, which disrupt pyrimidine synthesis (13).

In this study, the antioxidant capacity of mice serum receiving *T. chebula* extract was significantly higher than toxoplasmosis control group and positive control group. A dose of 100 mg/kg of *T. chebula* extract significantly reduced toxoplasma tachyzoites growth and survival of mice; it seems that the high antioxidant capacity of this extract (4.89±0.101 µg/mL) has a significant effect on decreasing oxidative stress. This effect may be related to high content of its phenol (276.66 ± 1.45 mg GAE/g) and flavonoid (39.99± 0.192 mg rutin equivalent/g dry extract) (38). However, in certain circumstances antioxidants may act as pro-oxidant that induces oxidative stress either by generation of ROS or by inhibiting antioxidant systems (39). Due to high antioxidant capacity of *T. chebula* extract, in 400 and 800 mg/kg dosages, this extract act as a pro-oxidant, so that the death rate of mice receiving these dosages was increased. Therefore, this extract up to a maximum dose of 200 mg/kg has been able to reduce oxidative stress and improve the disease with its antioxidant power. In this study, the level of serum MDA in toxoplasmosis control group was also determined. The level of serum MDA in toxoplasmosis control group was significantly higher than control groups. MDA serum levels in mice treated with 100 and 200 mg/kg of *T. chebula* extract were significantly lower than control group and toxoplasmosis control group. MDA is an organic compound with the formula $CH2 (CHO)_2$, and a byproduct of lipid metabolism in the body that its levels increase through fatty acid peroxidation. MDA can cross-link between enzyme activity and ion exchange permeability by affecting ion exchange through the cell membrane. In Turkey, higher levels of serum MDA were found in patients with toxoplasmosis compared with control group. This finding may be related to a decrease in the activity of the immune system in protecting tissue from free radicals.

This study also showed that toxoplasmosis like many other diseases can be related to oxidative stress (40). Therefore, plants, as a powerful source of antioxidants, can improve the process of oxidative stress-related diseases. The present study also showed a significant weight loss in liver and spleen of mice receiving *T. chebula* extract, compared with toxoplasosis control group. The anti-inflammatory effects of this herbal extract have caused weight loss of these organs. The anti-inflammatory effect of *T. chebula* extract in animal models was due to inhibition of nitric oxide synthesis (41).

Conclusion

T. chebula extract due to its high antioxidant power and reduced serum MDA levels as well as a good anti-inflammatory effect could be a suitable option for further studies. As one of the potential uses of herbal extracts with anti-toxoplasmic effect is their use in preventing congenital toxoplasmosis and toxoplasma reactivation in patients with immune deficiency, the medicinal herbs with anti- toxoplasma effects could be considered as a suitable alternative or supplement to prevent and treat toxoplasmosis.

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The results described in this paper were part of a student thesis.

Conflict of interest

The authors declare no conflicts of interest.

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