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ORIGINAL ARTICLE

Antiparasitic effects of *Zingiber officinale* (Ginger) extract against *Toxoplasma gondii*

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Summary
Zingiber officinale Roscoe, Ginger, has been used in folk medicine as a medicinal plant, as well as a spice and food in many countries. This research was carried out to evaluate the antiparasitic effect of ginger root extract (GE) and GE/F1 (fraction 1 obtained from GE) against *Toxoplasma gondii* (*T. gondii*) *in vitro* and *in vivo*. The effects of GE and GE/F1 against the proliferation of *T. gondii*-infected C6 cells and *T. gondii* were evaluated by several indicators such as an MTT assay, nuclear staining, immunofluorescence staining, apoptotic proteins and animal testing. GE/F1 strongly inhibited the proliferation of *T. gondii*-infected C6 cells and *T. gondii* in a dose-dependent manner compared with sulfadiazine. After *T. gondii* invasion, C6 cells induced the activation of caspase-3, bax, p53 and p21 related to programmed cell death, and GE/F1 effectively suppressed the expression of caspase-3, bax, p53 and p21 causing cell death of the infected host cells. In addition, INF-γ, and IL-8 levels, and the viability of *T. gondii*-infected mice treated with GE/F1 (500 μg/ml) were not changed or increased during the period of the experiment. These results demonstrate that GE/F1 not only induces anti-*T. gondii* effects causing the inactivation of apoptotic proteins in infected host cells through the direct inhibition of *T. gondii* but also has antiparasitic properties which inhibit inflammatory cytokine secretion *in vivo*.

Key words: apoptotic protein; C6 glioma cells; cytokine; p53; programmed cell death

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a protozoan parasite which causes infection of the central nervous system in HIV/AIDS patients and chronic toxoplasmosis in young children or adults with an impaired immune system (Luft and Remington 1992). *T. gondii* is a typical infectious organism that contains micro-organelles such as mitochondria, Golgi, micropore, rhoptries, dense granules, and micronemes (Joiner and Roos 2002). *T. gondii* causes infection stages such as the tachyzoites, bradyzoites and sporozoites in humans and animals, and also induces the progressive proliferation pathway jn two stages (the cyst stage and the oocyst stage) when it proliferates in host cells. *T. gondii* forms a parasitophorous vacuole membrane (PVM) after invading host cells. PVM not only provides *T. gondii* with nourishment but also protects *T. gondii* from acidification (Dubey et al. 1998). A child can be infected through placental infection which is transmitted to the foetus from the mother infected by *T. gondii*, and people may become infected by eating raw or undercooked meat and contagion by sporulated oocysts in soil or cat faeces.

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Typically, sulfadiazine, pyrimethamine, and atovaquone are used in the clinic to treat *T. gondii*, but their side effects are often evident. Until recently, studies of medicinal plants for the treatment of toxoplasmosis have been rare, and research on effective and new substances of relatively low toxicity is urgently needed. *Zingiber officinale* Roscoe, commonly known as ginger, has been widely used as an herbal medicine and in folk medicine, as well as a spice and food in many countries around the world. Furthermore, it has been used for the treatment of human diseases such as common cold, cough, dyspepsia, diarrhea, and headache particularly in Asia. Ginger has been reported to have gastro-protective effects (Yamahara et al. 1988), and inhibitory effects against nausea and vomiting (Ernst and Pittler 2000), and is also known to have strong anxiolytic and anti-emetic activities (Vishwakarma et al. 2002). Ginger induces extensive bioactivities including anti-inflammatory (Grzanna et al. 2005), anticancer (Shukla and Singh 2007), antioxidant (Jaqetia et al. 2003), and antimicrobial effects (Ficker et al. 2003). Its main chemical components are natural compounds such as gingerenones A, B, C (Endo et al. 1990), gingerdiol (Kikuzaki et al. 1992), 6-, 8-, 10-Gingerol (Hiserodt et al. 1998), paradol, shogaol (Ma et al. 2004), and essential oil (Singh et al. 2008). Recently, the 10-shogaol and 1-dehydro-6-gingerdione isolated from ginger partially activate the serotonin 5-HT_{1A} receptor which is widely expressed in the central nervous system (CNS) (Nievergelt et al. 2010). *Ginkgo biloba* L. and *Glycyrrhiza glabra* L. are known as *G. biloba*, and *G. glabra* respectively. The extract of *G. biloba* causes anti-inflammatory (Kotakadi et al. 2008), molluscicidal (Yang et al. 2008), antimicrobial (Mazzanti et al. 2000), and anticancer activities (DeFeudis et al. 2003). The combination treatment of ginger and *G. biloba* has been reported to cause neuroprotective effects (anti-anxiety effects and effects on the brain's learning power and memory) in various behavioral tasks (Hasenöhrl et al. 1998). In addition, this combination preparation also shows memory-enhancing effects in aged animals (Topic et al. 2002). *G. glabra* has a variety of biological effects such as anticancer (Kanazawa et al. 2003), antimicrobial (Gupta et al. 2008), and antiviral activities (Fiore et al. 2008), and its extract has been recently utilized as a food additive in the food industry. These researches not only indicate the various biological activities of medicinal plants but also demonstrate their physiological effects *in vitro* and *in vivo*.

An effective herbal medicine for the treatment of toxoplasmosis has not been developed yet, even though drugs such as sulfadiazine and pyrimethamine have been used as anti-*T. gondii* drugs in hospitals. For this reason, we investigated the anti-*T. gondii* effects of ginger, *G. glabra*, and *G. biloba* that have been used as medicinal plants and as food in many countries.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), antibiotics and trypsin-EDTA were purchased from Invitrogen Corporation (Gibco®, U.S.A). RPMI medium 1640, dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyl-2H-tetrazolium bromide; Thiazolyl blue), albumin bovine serum (BSA), phosphate buffered saline (PBS), 0.4% trypan blue solution, Hoechst 33342 and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A). ECL western blotting detection kit was purchased from Millipore Korea Co., Ltd. Silica gel 60 ($Kieselgel_{60}$, 230–400 mesh) was purchased from Merck Chemical Co., Ltd (Germany). The Bio-Rad protein assay kit was purchased from GenDEPOT Co., Ltd. (P.O. Box 454 Barker TX 77413 U.S.A). All other chemicals and reagents were purchased from Merck Chemical Co., Ltd (Germany) and Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A).

Preparation of extracts and fractions

The dried ginger, *G. glabra*, and *G. biloba* were provided by the Oriental Medical Center, Kyung Hee University (Seoul, Republic of Korea). The powdered roots of ginger (2 kg) and *G. glabra* (2 kg), and the powdered leaves of *G. biloba* (2 kg) were extracted using 4 l of methanol at room temperature for 24 h respectively. These extracts were filtered by filter paper and vacuum pump, and then were evaporated under reduced pressure using a concentrator in a vacuum at 35 °C. Among these extracts, ginger extract (GE) strongly inhibited the proliferation of C6 cells infected with *T. gondii* (*T. gondii*-infected C6 cells) and *T. gondii* compared with other extracts. GE was dissolved with methanol, and it was coated with silica gel. The coated GE was loaded onto a cotton wool pad at the top of the silica gel column and divided with various partitions by the silica gel column chromatography method using $CHCl_3$-MeOH eluate. All fractions were analysed by thin layer chromatography (TLC), and then we obtained nine fractions (column fractions obtained from GE) including GE/F1 (fraction 1 obtained from GE). Among these fractions, GE/F1 strongly inhibited the

proliferation and viability of *T. gondii*-infected C6 cells and *T. gondii*. Sulfadiazine (SF) was used as a treatment-positive group to evaluate whether or not GE/F1 has an anti-proliferative effect against *T. gondii*.

Animals

BALB-c/mice (4 weeks, n=150) were purchased from DaeHan Bio-Link Co., Ltd. Korea, and all animals were kept at 22±0.5 °C and 12 h-light/dark cycle in a controlled environment of a central animal care facility of the Kyung Hee University School of Medicine. Food and water were provided ad libitum. The facility was strictly maintained in accordance with National guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell lines and culture conditions of T. gondii

Rat C6 glioma cells (C6 cells) were purchased from Korean Cell Line Bank at Seoul National University, and Rat DI TNC1 normal brain cells (DI TNC1 cells) were purchased from American Type Culture Collection (U.S.A). Cells were cultured in RPMI medium 1640 containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg /ml streptomycin (Biofluids, Rockville, MD, U.S.A) in a humidified atmosphere containing 5% CO_2 in air at 37 °C. The RH strain of *Toxoplasma gondii* (*T. gondii*) was suspended with 1X PBS, which was injected in the abdominal cavity of each BALB-c/mouse. Three days after the injection, *T. gondii* was collected from the peritoneal fluids of each mouse kept in the abdominal cavities of the mice before use in the studies. In the *in vitro* study, C6 cells were infected with *T. gondii* (C6 cells: *T. gondii*=1:10).

MTT assay on the proliferation and cell viability

To evaluate the effects of ginger, *G. glabra*, and *G. biloba* extracts on the proliferation and viability of *T. gondii*-infected C6 cells, we investigated the viability of *T. gondii*-infected C6 cells exposed to these extracts. C6 cells were seeded in a 24 well plate (1×10^5 cells/well), which were infected with *T. gondii* (1×10^6 tachyzoites/well) after 24 h. The *T. gondii*-infected C6 cells were then incubated with varying concentrations (30–240 μg/ml) of ginger, *G. glabra*, and *G. biloba* extracts respectively, and their viability was determined by MTT assay. *T. gondii* was seeded in a 24 well plate (5×10^6/well), which was incubated with a different concentration (30–240 μg/ml) of GE, GE/F1, and SF for 24 h respectively. The PR (the proliferation rate) of *T. gondii*-infected C6 cells and *T. gondii* was calculated as follows: $OD_{control}–OD_{test\ sample}/OD_{control} \times 100\%$. Optical absorbance was measured at a wavelength of 570 nm using an ELISA leader.

Microscopic observation of T. gondii in C6 cells

C6 cells were seeded in a 24 well plate (5×10^4/well), which were incubated at 37 °C for 24 h. C6 cells were infected with *T. gondii* (5×10^5 tachyzoites/well), and then *T. gondii*-infected C6 cells were treated with 240 μg/ml of GE/F1 for 24 h. The morphological changes of C6 cells and *T. gondii*-infected C6 cells were observed under a light microscope (Nikon Eclipse TE 2000-U, Japan).

Nuclear staining of T. gondii-infected C6 cells

This assay was performed according to the method of Latt and Stetten (1976). Hoechst 33342 is a cell-permeable nuclear staining reagent which emits blue fluorescence when it combines to dsDNA. C6 cells were seeded in a 24 well plate (5×10^4 cells/well), which were infected with *T. gondii* (5×10^5 tachyzoites/well). The *T. gondii*-infected C6 cells were incubated with 120 μg/ml of GE/F1, and SF for 24 h respectively. After washing with 1X PBS, the *T. gondii*-infected C6 cells were fixed in 1X PBS containing 5% formaldehyde for 30 min, and then washed with 1X PBS and stained with a final concentration of 20 μM (Hoechst 33342) for 30 min in the dark. After staining, the cells were washed with 1X PBS three times, and their nuclei were observed under a UV fluorescent microscope (Nikon Eclipse TE 2000-U, Japan).

Immunofluorescence of PVM in T. gondii-infected C6 cells

C6 cells were seeded onto cover slips in a 24 well plate (5×10^4/well), which were infected with *T. gondii* (5×10^5 tachyzoites/well). *T. gondii*-infected C6 cells were incubated with 120 μg/ml of GE/F1 and SF for 24 h respectively. After washing with 1X PBS, they were fixed with 3% formaldehyde for 10 min and 0.05% (v/v) Triton X-100 for 5 min. The cells were blocked with 1X PBS containing 1% BSA for 1 h at room temperature after washing. A mouse monoclonal anti-PVM antibody was diluted with 1:100 (v/v) using 1% BSA/PBS, and then the cells were incubated with anti-PVM antibody solution at room temperature for 1 h. After washing, goat anti-mouse IgG-FITC-conjugated secondary antibody was diluted with 1:100 (v/v) using 1X PBS, which was added to each well. The cells were incubated at room temperature for 1 h, and were washed with 1X PBS three times. Their fluorescence was observed under a UV fluorescent microscope (Nikon Eclipse TE 2000-U, Japan).

Western blot analysis

C6 cells were incubated in a 6well plate (1×10^5/well) for 24 h, which were infected with *T. gondii* (1×10^6 tachyzoites/well). *T. gondii*-infected C6 cells were treated with different concentrations (60–120 μg/ml) of GE/F1 and SF for 24 h respectively, and harvested for the purpose of a western blot analysis. The pellets were lysed using RIPA lysis buffer (Elpis Biotech). The protein concentrations were measured at 595 nm using the Bio-Rad protein assay kit and 1% BSA as standard. Equal amounts of protein were loaded onto 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 1X PBS (5% skim-milk) containing 0.05% Tween-20 at room temperature for 1 h, and were also washed five times with 1X PBS containing 0.05% Tween-20. The membranes were incubated with the corresponding primary antibodies to p53 (1:500), p21 (1:500), caspase-3 (1:500), bax (1:500) and β-actin (1:2,000) respectively, overnight at 4 °C. After the membranes were washed with 1X PBS containing 0.05% Tween-20, they were incubated with goat anti-mouse, goat anti-rabbit or rabbit anti-goat IgG HRP conjugated secondary antibodies at room temperature for 2 h. After repeating the washing step, protein bands were visualized using the ECL western blotting analysis kit (Millipore Corporation, Billerica, MA 01821 U.S.A). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA), and R&D System (Minneapolis, MN, U.S.A).

The viability of T. gondii-infected mice

Sixty animals were divided into control (n=15) and experimental groups (3 groups, n=45). *T. gondii* was seeded in a 12 well plate (1×10^7/well), which was incubated with 250, and 500 μg/ml of GE/F1 for 24 h. *T. gondii* treated with 250 and 500 μg/ml of GE/F1 was harvested before the injection in the abdominal cavity of each mouse in the experiment group, which was washed with 1X PBS three times. The pellets were also suspended with 1X PBS, which were injected in the abdominal cavity of each BALB-c/mouse in the experimental groups. Mice infected with *T. gondii* (*T. gondii*-infected mice) were used as an infection positive group, and all animals were kept in a central animal care facility during the experiment.

Measurements of IFN-γ, and IL-8

Ninety animals were divided into control (n=30) and experimental groups (2 groups, n=60). *T. gondii* was seeded in a 12 well plate (1×10^7/well), which was incubated with 500 μg/ml of GE/F1 for 24 h. *T. gondii* treated with 500 μg/ml of GE/F1 was harvested before the injection into the abdominal cavities of the mice, and it was washed with 1X PBS three times. The pellets were also suspended with 1X PBS, which were injected into the abdominal cavity of each BALB-c/mouse in the experimental groups. The *T. gondii*-infected mice were used as an infection positive group. Blood samples were collected from the left ventricle of heart of the anaesthetised mice into heparinized syringes every day for 5 days after the injection. Serum was separated by centrifugation at 12000 rpm for 15 min at 4 °C, and stored in vials at –70 °C until analysis for IFN-γ, and IL-8. IFN-γ levels were measured with commercial sensitive ELISA kits (SABiosciences, QIAGEN Company, MD 21703, USA), and IL-8 levels were measured with commercial sensitive ELISA kit (COSMO BIO CO., LTD. Koto-ku, Tokyo 135-0016, Japan). All other chemicals and reagents were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A). The activity of IFN-γ, and IL-8 in serum was measured according to the manufacturer's protocol.

Statistical analysis

All results were expressed as mean ± S.E.M. Statistical analysis of the data was performed using Student's *t*-test and one-way analysis of variance (ANOVA software version 14). The significance level was $2\alpha=0.05$.

RESULTS

Effect of GE and GE/F1 on the proliferation of T. gondii-infected C6 cells and T. gondii

T. gondii has various micro-organelles such as mitochondria and ER network. MTT is an established assay performed in many laboratories. We evaluated the effects of ginger, *G. glabra* and *G. biloba* extracts on the cell proliferation and cell viability of *T. gondii* using an MTT assay. After *T. gondii*-infected C6 cells were incubated with various concentrations (30–240 μg/ml) of the extracts for 24 h, their viability was decreased in a dose- and time-dependent manner (Fig. 1). In particular, GE strongly inhibited the proliferation and viability of *T. gondii*-infected C6 cells compared with other extracts. *T. gondi* -infected C6 cells treated with 240 μg/ml of GE/F1 for 24 h exhibited antiproliferation including *T. gondii* fragmentation and a significant decrease in *T. gondii*, as well as morphological changes including cell shrinkage, membrane blebs, and cell fragmentation compared with *T. gondii*-infected C6 cells (Fig. 2). As shown in Figs 1–3, GE/F1 effectively inhibited the

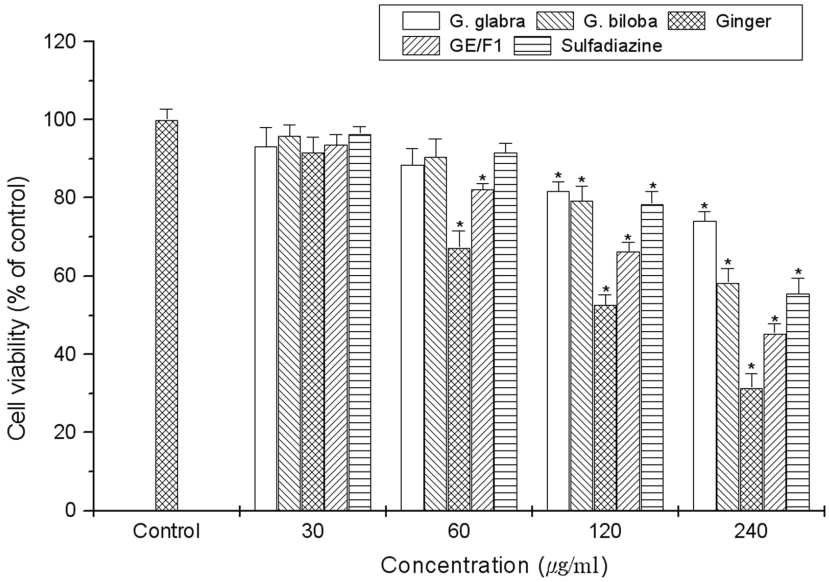

Fig. 1. **The inhibitory effect of different extracts on the viability and proliferation of *T. gondii*-infected C6 cells**. *T. gondii*-infected C6 cells were incubated with concentrations (30–240 μg/ml) of ginger, *G. glabra*, *G. biloba* extracts, GE/F1, and SF for 24 h, respectively. Results are expressed as a percentage of the control, and all data are presented as mean ± S.E.M. * Statistically significant as compared with control.

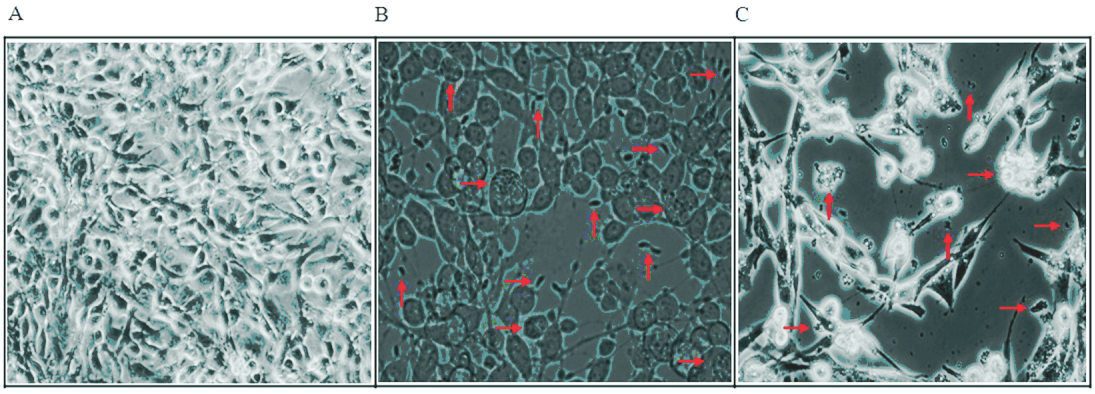

Fig. 2. **Morphological changes of *T. gondii*-infected C6 cells.** *T. gondii*-infected C6 cells were incubated with 240 μg/ml of GE/F1 for 24 h. (A) Uninfected C6 cells. (B) *T. gondii*-infected C6 cells (the arrow shows tachyzoites into PVM formed by proliferation of *T. gondii* in C6 cells after *T. gondii* invasion, and *T. gondii*). (C) *T. gondii*-infected C6 cells treated with 240 μg/ml of GE/F1 (the arrow shows *T. gondii* fragmentation, and the morphological changes including cell shrinkage, membrane blebs).

viability of *T. gondii*-infected C6 cells and *T. gondii* compared with SF, which induced the *T. gondii*-selective growth inhibitory effect against *T. gondii*, and then PR was measured less than 45% at concentrations of 240 μg/ml (Fig. 3). The 50% inhibitory concentration (IC_{50}) values of GE, GE/F1, and SF against *T. gondii* were measured as 220.83, 205.56, and 276.81 μg/ml, respectively. These results indicate that GE/F1 effectively caused anti-proliferation of *T. gondii*-infected C6 cells through the direct inhibition of *T. gondii*. On the other hand, GE/F1 showed no obvious cytotoxicity against DI TNC1 cells (Fig. 4).

Inhibitory effect of GE/F1 on PVM formed by T. gondii proliferation in host cells

T. gondii has distinguishing features such as anti-apoptosis through the inactivation of apoptotic proteins during the early- and intermediate proliferative phase in host cells after cell invasion. The activation of PVM is accelerated in the intermediate stage (the stage before release of

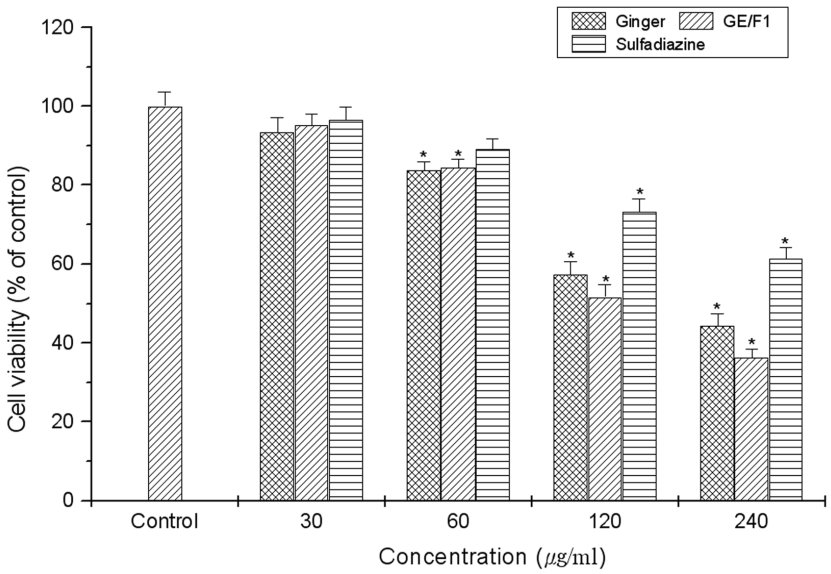

Fig. 3. **The effects of GE, GE/F1, and SF on the proliferation of *T. gondii*.** *T. gondii* was incubated with concentrations (30–240 μg/ml) of GE, GE/F1, and SF for 24 h, respectively. Results are expressed as a percentage of the control, and all data are presented as mean ± S.E.M. Symbols as in Fig. 1.

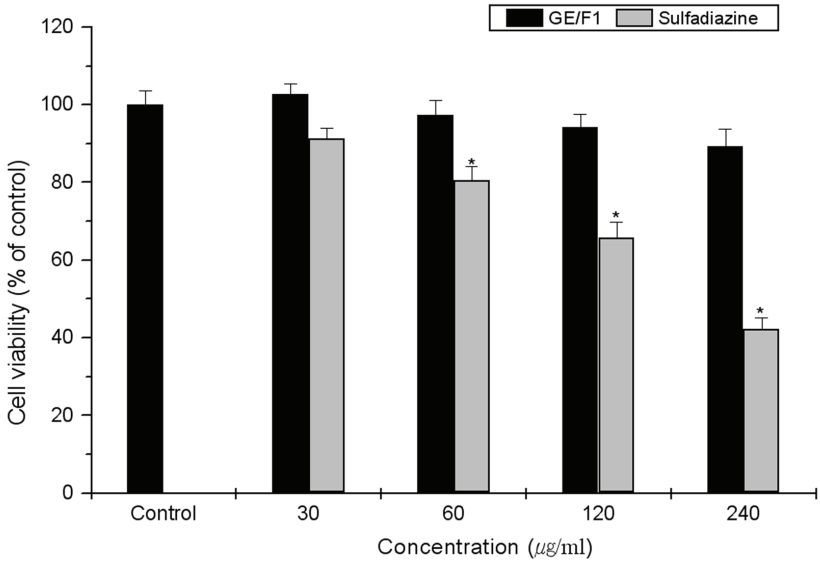

Fig. 4. **Cytotoxicity of GE/F1 and SF on the viability of normal cells.** DI TNC1 cells were treated with different concentrations (30–240 μg/ml) of GE/F1 and SF for 24 h respectively. Results are expressed as a percentage of the control, all data are presented as mean ± S.M.E. Symbols as in Fig. 1.

T. gondii from PVM) of *T. gondii* in a time-dependent manner. We evaluated the antiproliferative response of *T. gondii* induced by interaction between PVM and GE/F1 during the proliferation stage of *T. gondii* in host cells. The morphological change of PVM and nuclei of *T. gondii* was markedly decreased in *T. gondii*-infected C6 cells treated with 120 μg/ml of GE/F1 and SF respectively (Fig. 5). These results show that GE/F1 selectively inhibited the proliferation of *T. gondii* and PVM formed by *T. gondii* invasion in infected host cells.

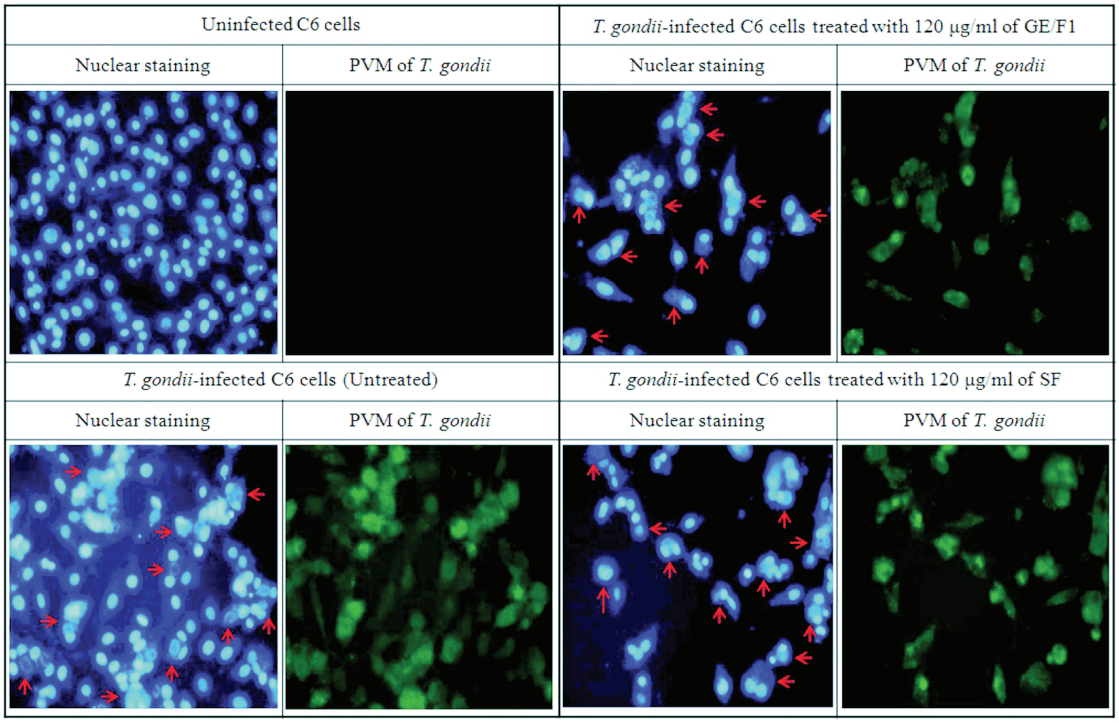

Fig. 5. **Nuclear staining and immunofluorescence of *T. gondii*-infected C6 cells treated with GE/F1 and SF.** C6 cells were seeded onto cover slips in a 24 well plate (5×10^4/well), which were infected with *T. gondii* (5×10^5/well) after 24 h. *T. gondii*-infected C6 cells were incubated with 120 μg/ml of GE/F1 and SF for 24 h, respectively. Green shows morphological changes of PVM formed by *T. gondii* proliferation in C6 cells after *T. gondii* invasion. Fluorescence simultaneously shows nucleus of *T. gondii* in PVM, and nucleus of C6 cells respectively (the arrow shows the nucleus of *T. gondii* into PVM formed by proliferation of *T. gondii* in C6 cells).

Modulation of GE/F1 on apoptotic proteins in T. gondii-infected C6 Cells

The caspase-3, bax, p53 and p21 proteins play crucial roles in the regulation of cell cycle progression during cell division (Morgan 1995). In this study, we evaluated the inhibitory effects of GE/F1 on the apoptotic signalling proteins which cause cell cycle arrest and apoptosis during the proliferation stage of *T. gondii* in *T. gondii*-infected C6 cells. The p53 is a tumor-suppressor protein of the cell death pathway, and the p21 functions as a regulator of cell cycle progression at G_1/S or G_2/M transition in response to a variety of stress stimulation. The caspase-3 and bax are indicator proteins of the mitochondria pathway of the cell death mechanism. The expression of p53, p21, caspase-3 and bax activated by intracellular proliferation of *T. gondii* was markedly decreased in *T. gondii*-infected C6 cells treated with GE/F1 compared with both *T. gondii*-infected C6 cells treated with SF and *T. gondii*-infected C6 cells (*T. gondii* infection positive group) (Fig. 6). These results suggest that GE/F1 effectively blocked apoptosis of C6 cells induced by *T. gondii* invasion through the direct inhibition of *T. gondii*.

Effects of GE/F1 on the viability and proliferation of T. gondii in T. gondii-infected mice

T. gondii is a parasite which induces serious infectious diseases such as brain injury and immune deficiency in human and animals. For this reason, we evaluated the antiproliferative effect of GE/F1 against the proliferation and growth of *T. gondii* in *T. gondii*-infected mice. The mice were observed during the period of the experiment after the injection of *T. gondii* treated with 250 and 500 μg/ml of GE/F1 for 24 h. As mentioned above, *T. gondii*-infected mice treated with 500 μg/ml of GE/F1 showed higher viability than *T. gondii*-infected mice and *T. gondii*-infected mice treated with 250 μg/ml of GE/F1, and the mice maintained the same activity and dynamics as the control group. In addition, there was a significant difference of the survival rate between *T. gondii*-infected mice and *T. gondii*-infected mice treated with GE/F1 (Fig. 7). These results demonstrate that GE/F1 has anti-*T. gondii* effects and unique properties which inhibit the proliferation and growth of *T. gondii*.

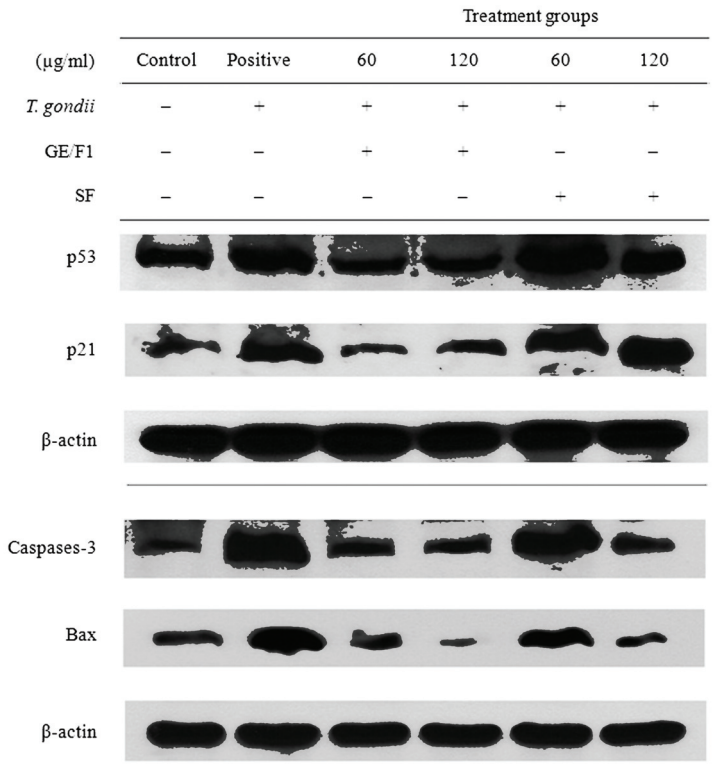

Fig. 6. **Change of apoptotic signalling proteins of *T. gondii*-infected C6 cells.** *T. gondii*-infected C6 cells were treated with different concentrations (60–120 μg/ml) of GE/F1 and SF for 24 h respectively, and protein expression was measured using western blot analysis.

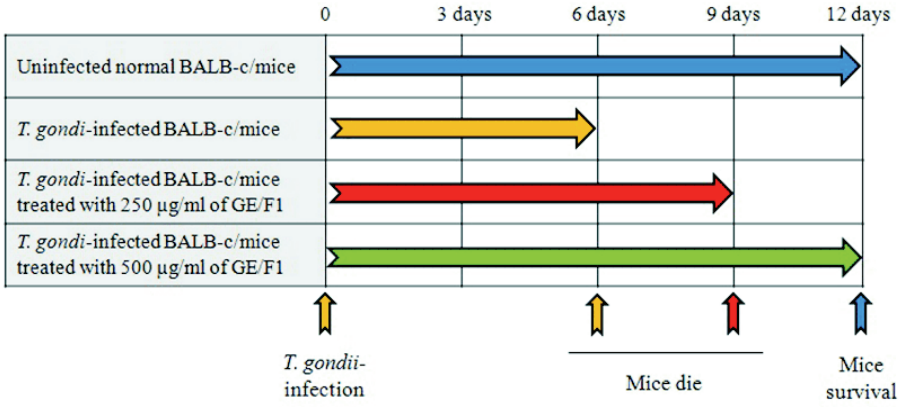

Fig. 7. **Effect of GE/F1 on the viability of *T. gondii*-infected mice.** *T. gondii* was incubated with 250 and 500 μg/ml of GE/F1 for 24 h, and was injected into the abdominal cavity of each mouse in the experimental groups. Sixty animals were divided into control (n=15) and experimental groups (3 groups, n=45).

Changes of cytokines in T. gondii-infected mice

Cytokines play in endocrine via specific cell surface receptors on their target cells as key players in the regulation of the immune response. They exert their actions during tissue damage leading to inflammation and infections such as parasites, bacteria, virus, and endocrinological autoimmune diseases. Particularly, the release of cytokines such as IFNs (INF-α, β, γ), and IL-8 which are produced by infections and inflammation is an important component of the

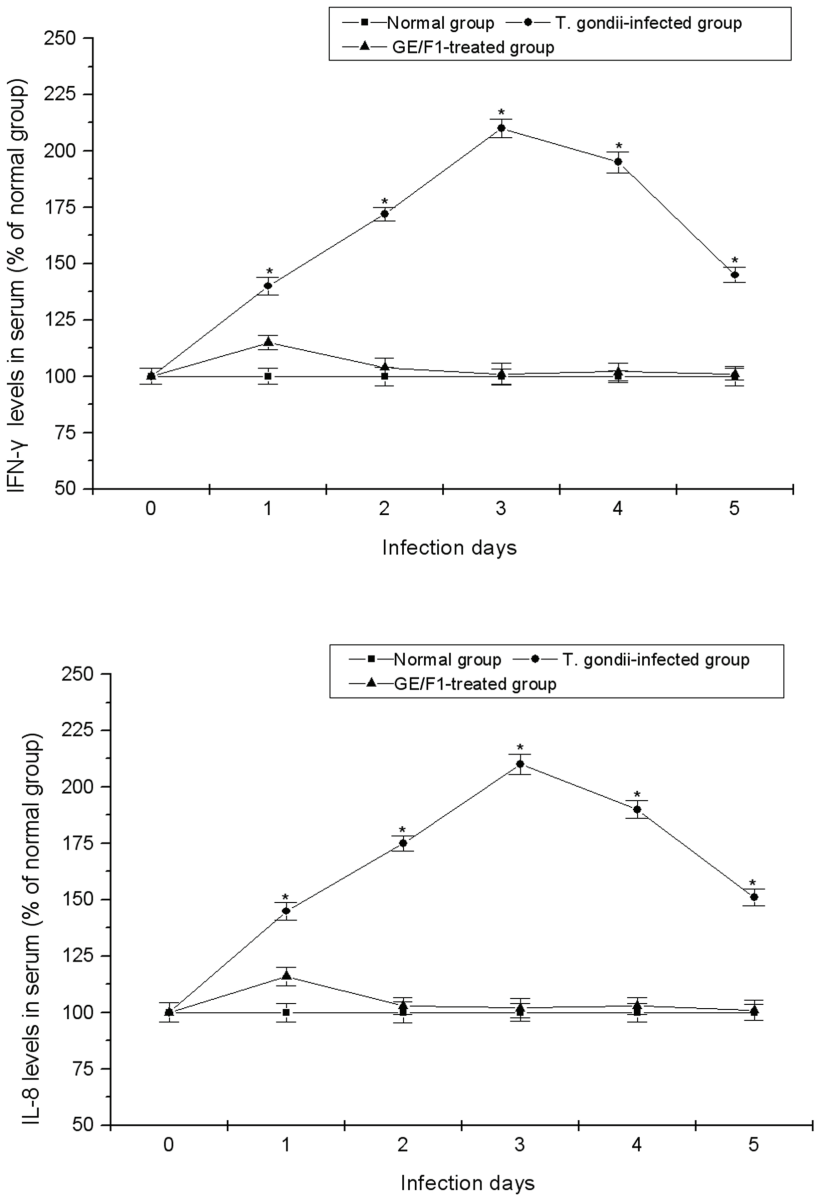

Fig. 8. **The serum levels of IFN-γ, and IL-8 of *T. gondii*-infected mice treated with GE/F1.** The serum levels of (A) IFN-γ, and (B) IL-8. *T. gondii* was treated with 500 μg/ml of GE/F1 for 24 h, and was injected into the abdominal cavity of each mouse in the experimental group. The cytokines were measured using ELISA kits in a time-dependent manner. Results are expressed as a percentage of the control, and all data are presented as mean ± S.M.E. * Statistically significant as compared with control.

immune response to secrete cytokines that help to activate endocrine system. As shown in Fig. 8, although secretions of INF-γ, and IL-8 were slightly increased on first day after the injection of *T. gondii* treated with 500 μg/ml of GE/F1, their levels were not increased during infection periods. On the other hand, the concentrations of cytokines in the serum of *T. gondii*-infected mice were rapidly increased compared with the control group. These results demonstrate that GE/F1 effectively inhibited the secretions of INF-γ and IL-8 which are rapidly activated by infections, inflammation, and tissue damage in endocrine system of mice.

DISCUSSION

T. gondii causes serious complications in AIDS/HIV patients, as well as parasite symptoms into the brain through extrinsic infection. *T. gondii* causes spontaneous abortion in pregnant women and foetal infection through the placenta during pregnancy. It is also one of the important parasites which induce zoonosis. When host cells are infected by a parasite, the host cells activate protective systems including the immune-response and defensive mechanism against parasitic invasion and stimulation of secretion released from the parasite. Particularly, IFN-γ produced by helper T-cells accelerates the differentiation of T lymphocytes through the signalling transport of the STAT1 and T-bet, and then they activate anti-protozoa system which causes the resistance to *T. gondii* invasion *in vivo*. However, *T. gondii* inhibits the production of IL-4, the activity of caspase-3 and the release of cytochrome-*c* from mitochondria after invasion in host cells. *T. gondii* suppresses apoptotic signals of host cells to induce anti-apoptosis during its proliferation stage in host cells (Laliberte and Carruthers 2008). In this study, we evaluated the effect of GE/F1 which inhibits the proliferation of *T. gondii* and the changes of cytokines induced by *T. gondii* infection through animal testing, and signalling pathways of apoptotic proteins in host cells after *T. gondii* infection.

In general, apoptotic signalling pathways induce apoptosis through the G_1/S or G_2/M transition of the cell cycle by regulating the expression of genes. Among apoptotic proteins, bax and caspase-3 are important indicators related to the mitochondrial signalling pathways that regulate the activation of the apoptotic cascade mechanism, and their activity results in the subsequent apoptotic progression (such as caspase-9, -7, -6, Cyto-*c*, and CAD). The overexpression of p53 induces an increase of bax and p21 expression, and a suppression of bcl-2 expression (Agarwal et al. 1998). Furthermore, p21 is a CKI that inhibits the activity of Cyclin-Cdk complex at the G1 or G2 checkpoints of cell cycle, and the expression of p21 is controlled by tumour suppressor p53 at G_1 phase (El-Deiry et al. 1993, Harper et al. 1993, Gartel and Radhakrishnan 2005, Choi et al. 2011). These apoptotic proteins promote cell death and cell arrest at the cell cycle. The spontaneous apoptosis of *T. gondii*-infected cells is induced by the activation of caspase-3, -7, -8 and -9 during the early apoptotic stage of host cells after *T. gondii* invasion (Laliberte and Carruthers 2008). However, *T. gondii* exerts influence on the apoptotic progress and cell arrest pathways of host cells after cell invasion. They also proliferate using a three-phase cycle (S phase, late S phase and G1 phase) which comprises 50–70% of the parasite doubling time. *T. gondii* induces inactivation of cell cycle initiator (cyclin-cdk complex and p21) and apoptotic mediator (caspase families) through its signalling pathways during the early- and intermediate proliferative phase of *T. gondii* in host cells. Furthermore, activation of the PI3K (phosphoinositol 3 kinase) in infected cells leads to inactivation of Bad and the inhibition of the forkhead transcription factor (FKHR1) in the host cells (Gubbels et al. 2008). In the present study, the proliferation of *T. gondii* and PVM were remarkably inhibited in *T. gondii*-infected C6 cells treated with GE/F1 compared with *T. gondii*-infected C6 cells (Fig. 5). Although the expression of bax, caspase-3, p53 and p21 was increased in C6 cells after *T. gondii* infection, their expression was decreased in a concentration-dependent manner in infected cells after treatment with GE/F1 (Fig. 6). On the other hand, both p53 and p21 were significantly increased in *T. gondii*-infected C6 cells and *T. gondii*-infected C6 cells treated with SF compared with uninfected C6 cells.

The immune system *in vivo* recognizes the presence of pathogens by several proteins and cytokines. Cytokines such as IFN-γ and IL-8 play pivotal roles in a variety of immune responses of the B-cells, T-cells, NK cells, mast cells, and macrophages which are activated by tissue damage, inflammation or infection of pathogens such as bacteria, virus, and parasites. They are chemical signals which are produced by macrophage, epithelial and endothelial cells at the site of inflammation. We evaluated the changes of cytokines in the serum of *T. gondii*-infected mice. After the injection of *T. gondii* treated with 500 μg/ml of GE/F1, the concentrations of INF-γ, and IL-8 in serum were not increased during the period of parasitic infection compared with infection positive group. Even though cytokine levels of *T. gondii*-infected mice treated with 500 μg/ml of GE/F1were slightly increased on the first day after injection, the increase of cytokines may be a low response of the immune network induced because immune cells recognize their presence as foreign substances. In addition, *T. gondii*-infected mice treated with GE/F1 (500 μg/ml) showed the same viability as the control group during the experiment. Taken together, these results indicate that *T. gondii*-infected host cells induce activation of the cell cycle initiator (p21) and apoptotic mediator (p53, caspase-3 and bax) which cause host cell apoptosis during the proliferative phase (the stage before the release of *T. gondii*) of *T. gondii*. The results show that *T. gondii* increases inflammatory cytokine secretion *in vivo* after its infection.

In summary, the results of this study demonstrate that GE/F1 not only has anti-*T. gondii* effects causing the direct inhibition of *T. gondii* but also inhibits the activation of apoptotic proteins (p53, p21, caspase-3, and bax) induced by *T. gondii* proliferation in infected host cells. The results also indicate that GE/F1 has antiparasitic properties which are able to maintain the survival of *T. gondii*-infected mice. Therefore, our research findings provide substantial evidence that GE/F1 can be used as a new candidate substance for anti-*T. gondii* drug development through the blocking of apoptotic proteins *in vitro* and inflammatory cytokine inhibition *in vivo*, and that, in general, extracts and substances derived from medicinal plants may be used as useful medicinal resources in the development of antiparasitic drugs.

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