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Effects of *Gentiana scabra var. buergeri* Extract on Toxoplasmastic Activity of Macrophages

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Gentiana scabra var. buergeri (*G. scabra*) is a herb known to have therapeutic effect in infection diseases. We studied cellular activation and antitoxoplasmosis in macrophages after *G. scabra* stimulation. Macrophage activation was detected by nitrite production. Macrophages were treated with *G. scabra* extracted with water or methanol. Maximal nitrite production was detected in macrophages after stimulation of *G. scabra* extract 0.1 mg/ml. Maximal nitrite concentration was 23.22 0.003 uM/L in macrophages after water extract of *G. scabra* and was 24.07 1.41 uM/L after methanol extract of *G. scabra*. Effect of *G. scabra* in the phagocytic capacity of macrophages was monitored by using PI (percentage of macrophage infected by *T. gondii*) method. The minimum PI (42.5 2.31) was detected in macrophages treated by water extract of *G. scabra* 0.1 mg/ml before infection of *T. gondii*. We also examined toxoplasmastatic capacity of macrophage using FI (fold increase) method. The minimum FI (4.46 1.16) was shown in macrophages after water extract of *G. scabra* 0.1 mg/ml pretreatment before infection. Under electron microscope, proliferation of *T. gondii* was inhibited by extract of *G. scabra* treatment in macrophages and the mitochondrion and lysosomal vacuoles within cells were increased. Taken together, *G. scabra* extract activates macrophages and induces toxopalsmastatic activity after *T. gondii* infection. It is suggested that *G. scabra* may be used as a therapeutic drug against toxoplasmosis.

Key Words: *Gentiana scabra var. buergeri*, *Toxoplasma gondii*, Macrophage, Toxoplasmosis

INTRODUCTION

Many herbs are used as therapeutic agents against parasitosis by immunomodulation[11,17,21,23,24]. Quinine extracted from bark is a therapeutic agent which is effective against malaria. The extracts of *Digenera simplex*, *Sementorreyz* and *Aspidium* are known as therapeutic agents against teaniasis[24]. Natural herbs are widely used as medicine against infectious diseases in Oriental medicine. Recently, Soh *et al.* (1996)[24] reported the extract of *Coix lacryma* (*C. larcryma*) has antitoxoplasmosis and activates macrophage. It was demonstrated that the extract of *Artemisia annua* inhibited the proliferation of *Eimeria tenella*[21]. In rats treated with the extract of *Artemisia annua* and *Amaranthus mangostanus*, it showed significant inhibition on *Cryptosporidium parvum* growth[20].

Toxoplasma gondii is an obligate intracellular protozoan parasite with global distribution among humans and animals. Under normal conditions, *T. gondii* infection is largely asymptomatic, but in innate infection, it can cause disease including hydrocephalus and choroiditis[3]. In individuals who are immunocompromised, such as patients with AIDS it can become widely disseminated, causing severe toxoplasmosis or encephalitis after infection[25,26]. Patients in St. Mary's Hospital were examined by indirect latex agglutination test in order to evaluate anti-toxoplasma antibody. Patients with positive results was 4.3%[3]. Mental patients hospitalized in National Seoul Mental Hospital were 1.3 percent of positive titers in anti-toxoplasma antibody[4]. It indicates that toxoplasma infection is widespread in Korea.

No drugs are completely therapeutic agents until now since drugs against toxoplasmosis have toxicity, drug-tolerance and side effects[9]. Pyrimethamine-sulfamethoxazole

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and Clindamycin-sulfadiazine are known as effective drugs against toxoplasmosis but it was reported that therapeutic effect is markedly decreased in pregnant women[9,18]. New drug having significant therapeutic effect and no side effects was needed for toxoplasmosis therapy.

Gnetiana scabra is used as a therapeutic agent against bacterial, fungal, and parasitic diseases in Korean oriental medicine and is a perennial herb involved in Gentianaceae and lives in several mountains in Korea. Pharmacological and therapeutic effect against infectious disease is not well known. We studied whether *G. scabra* has inhibitory effect on *T. gondii* infection and proliferation in macrophages.

MATERIALS AND METHODS

1. Materials

Dulbecco's modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Life Technologies, Inc. (Gaithersburg, MD). Other materials were obtained from Sigma (St. Louis, MO).

2. Parasite

RH strain of *T. gondii* was maintained in ICR mice. *T. gondii* was used for infection after extraction from the peritoneal exudates with sterile syringe as described previously[6].

3. Experimental animals

ICR mice weighing about 17 to 20 g at 6 to 7 weeks old were obtained from Korean Experimental Animal Center. Female ICR mice were used throughout the experiments.

4. Preparation of *G. scabra* extract

The trunk of *G. scabra* was dissolved with 70% methanol or distilled water, heated, cooled and filtered. The extract was made by lyophilization at -40C. It was dissolved with 0.01 M Tris-HCl (pH 7.4), filtered through a membrane filter (0.22 um) and diluted to the concentration of 50 mg/ml, 40 mg/ml, 3 warmed ml, 20 mg/ml and 10 mg/ml. The storage of *G. scabra* extract was stored at 4'C.

5. Isolation of macrophages

Macrophages were harvested from mice which were inoculated i.p. 3 day previously with 2 ml of 3% thioglycollate. Cells were harvested by injecting RPMI into the peritoneal cavity and plated at 1106 per well in 24 well plates. After 6 hrs incubation in CO_2 incubator at 37C, macrophages were washed with sterile phosphate buffered saline (PBS) for removing suspension cells and added with RPMI containing 10% FBS.

6. Nitrite measurement

Nitrite (NO_2) levels were used as an indicator of reactive nitrogen intermediates in samples and were measured by the Griess assay[10]. In brief, 100-l aliquots of supernatant of macrophages after IFN or LPS or *G. scabra* extract treatment were added to 96-well plates followed by a 100l of a 1 : 1 mixture of 1% sulfanilamide dihydrochloride in 2.5% H_3PO_4 and 0.1% naphthylethylenediamide dihydrochloride in 2.5% H_3PO_4. After 10 min incubation at room temperature, the absorbance of the samples (A540) was read spectrophotometrically and concentration of nitrite was determined by comparison with a standard curve generated with sodium nitrite ($NaNO_2$).

7. Effect of *G. scabra* on *T. gondii* infection and proliferation

Macrophages were plated at 1106 per well in 24 well plates and incubated with presence or absence of IFN-r or LPS or *G. scabra* extract for 48 hrs. Cells were infected with *T. gondii* at 1106 per well for 30 min. After 48 hr incubation, cells were fixed and stained with modified Giemsa dye. PI was calculated as percentage of macrophage infected with *T. gondii* and FI was calculated as ratio of the mean number of *T. gondii* per 100 macrophages after 48 hr incubation to the mean number of *T. gondii* per 100 macrophages at the beginning of the assay.

8. Transmission electron microscopy

Isolated macrophages plated at 1106 per well. After 12 incubation, cells were treated with *G. scabra* extract (0.1 mg/ml) following 12 and 24 hr incubation respectively. Cells were fixed with 2.5% glutaraldehyde, washed with PBS, postfixed with 1% osmium tetraoxide (pH 7.4), dehydrated in ethanol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope.

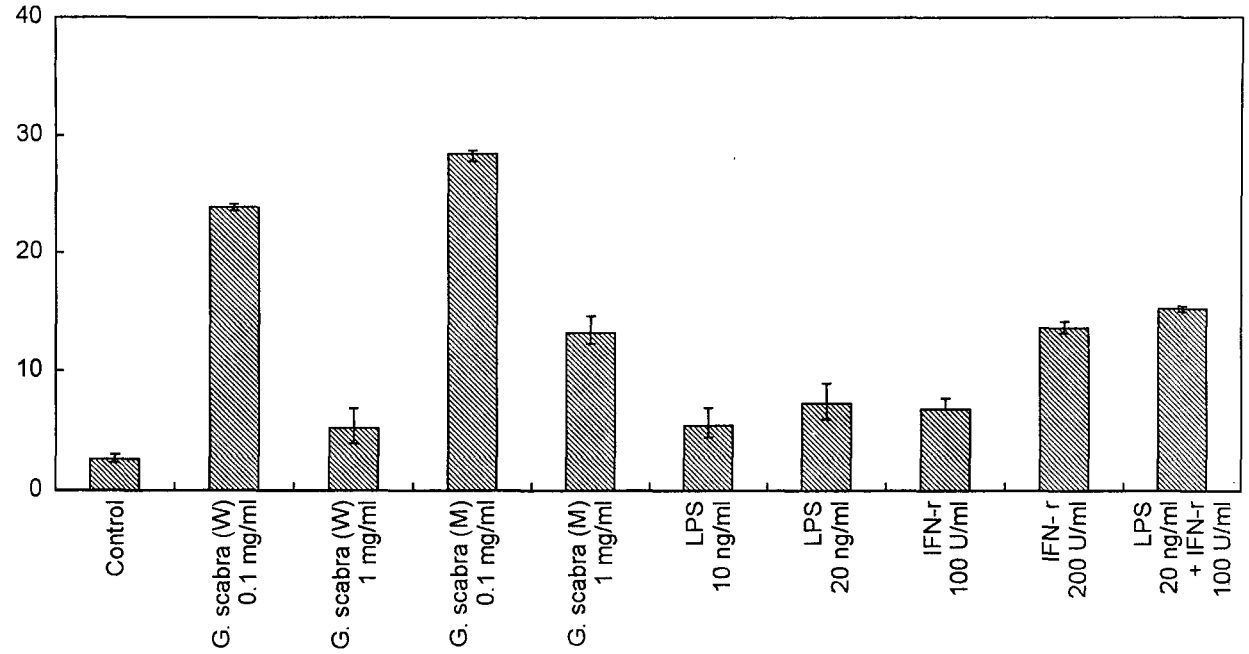

Fig. 1. Nitrite production of macrophages after *G. scabra* extract treatment.

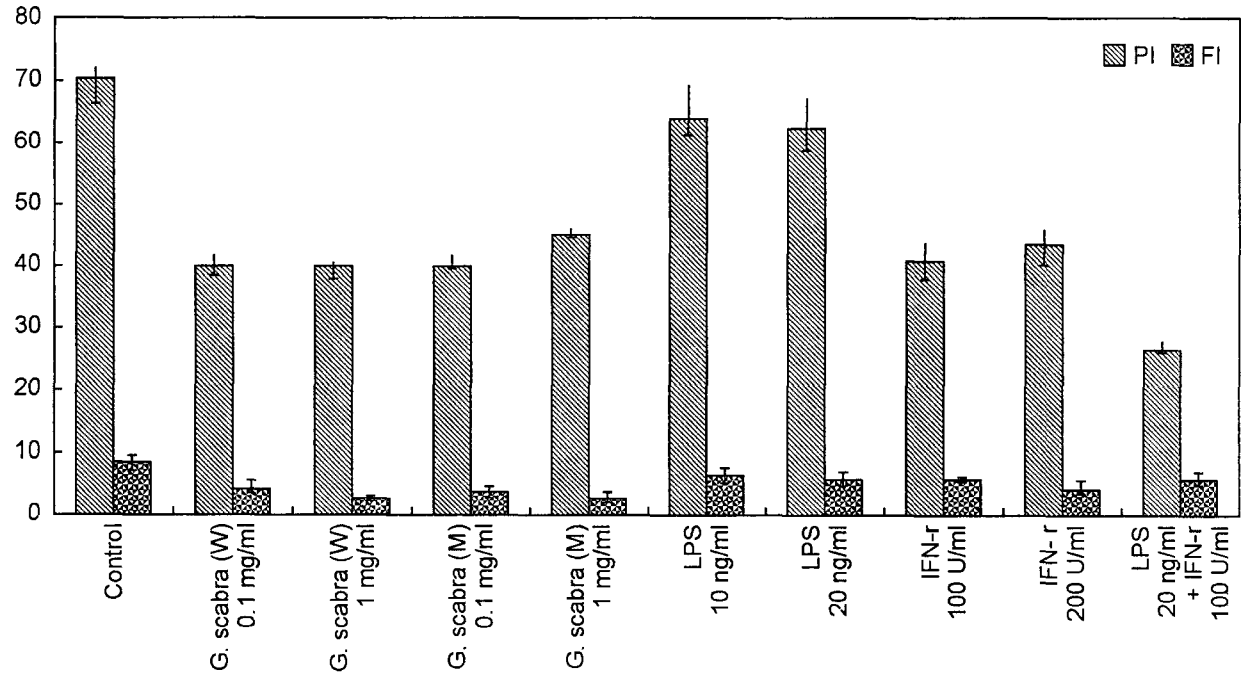

Fig. 2. PI (percentage of macrophage infected *T. gondii*) and FI (fold increase) in macrophages after *G. scabra* extract treatment.

RESULTS

1. Nitrite production of macrophages stimulated with *IFN-r and LPS*

Nitrite concentrations of 6.06 1.64 (100 U/ml) and 13.06 1.65 uM/L (200 U/ml) were produced in macrophages after IFN-r treatment. LPS activates macrophage production, followed induced nitrite concentration of 4.64 2.41 uM/L (10 ng/ ml) and 6.74 3.95 uM/L (20 ng/ml). Macrophages produced more nitrite production after LPS and IFN-r treatment than individual treatment (Fig. 1).

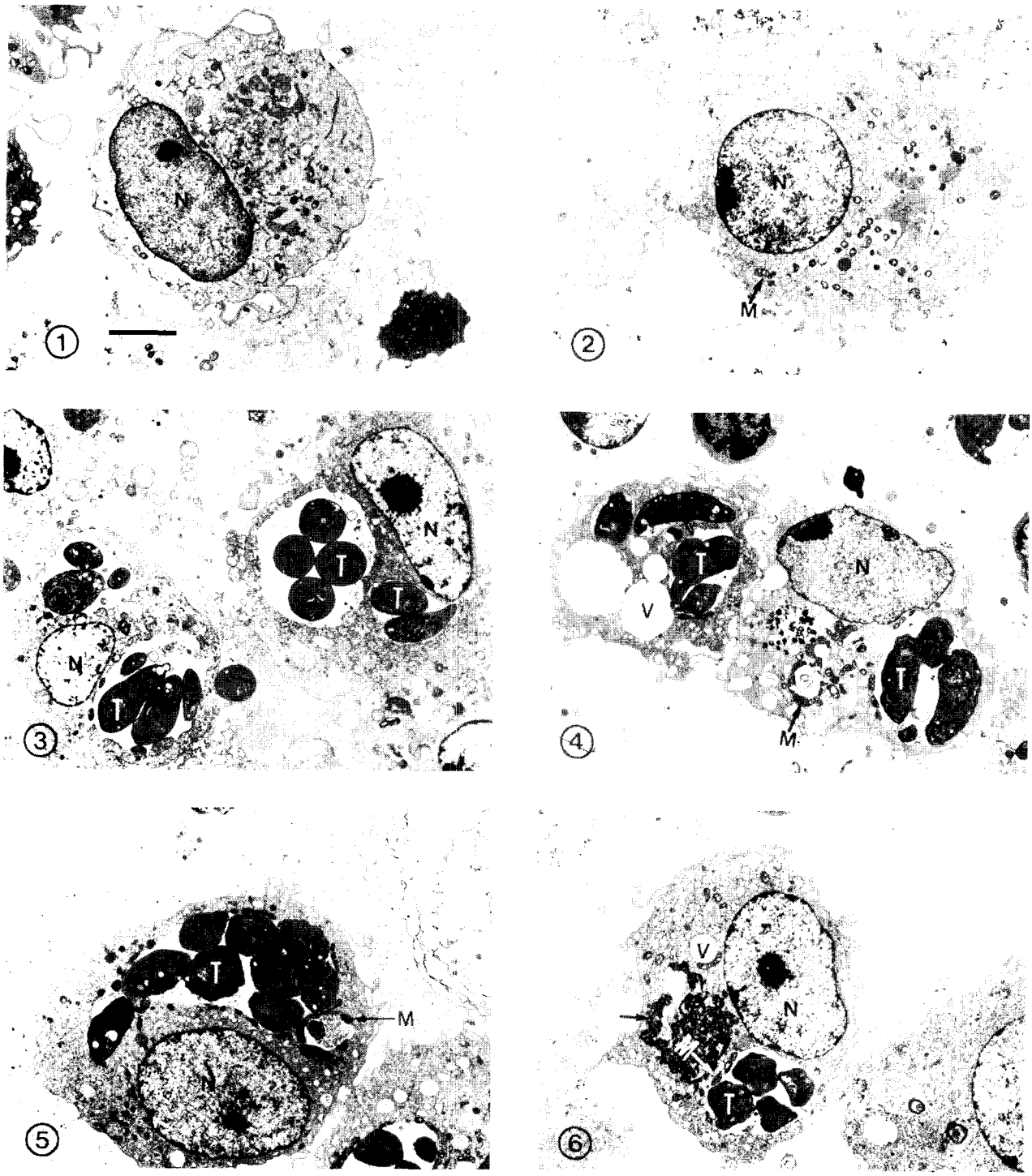

Plate 1. Electron micrograph of macrophage N, nucleus. ×3,000, scale bar = 2 μm. **Plate 2.** Electron micrograph of macrophage after *G. scabra* extract treatment M, mitochondria. **Plate 3.** Electron micrograph of macrophage incubated for 12 hr after *T. gondii* infection T, *T. gondii*. **Plate 4.** Electron micrograph of macrophage that was infected with *T. gondii* after *G. scabra* extract treatment and subsequently incubated for 12 hr V, vacuole. **Plate 5.** Electron micrograph of macrophage incubated for 24 hr after *T. gondii* infection. **Plate 6.** Electron micrograph of macrophage that was infected with *T. gondii* after *G. scabra* treatment and subsequently incubated for 24 hrs.

2. Nitrite production of macrophages after *G. scabra* extract treatment

After water extract of *G. scabra* treatment (0.1 mg/ml), nitrite production was shown at the concentreation of 23.22 0.03. In macrophages treated with methanol extract of *G. scabra* (0.1 mg/ml), the concentreation of nitrite production was 24.07 1.41 uM/ml. *G. scabra* extract induced nitrite production in macrophages as much as pyrimethamine used as therapeutic drug against *T. gondii* (Fig. 1). This results indicates *G. scabra* extract activates macrophages.

3. PI (percentage of macrophage infected *T. gondii*) and FI (fold increase) in macrophage stimulated with LPS and IFN-r

Macrophages were treated with LPS or IFN-r, infected with *T. gondii*, incubated for 48 hr and stained with Giemsa dye. We calculated PI and FI as described materials and methods. PI in LPS (20 ng/ml) treatment was 63.13 6.75 and FI was 4.57 0.97. PI in IFN-r (100 U/ml) treatment was 42.24 4.23 and FI was 2.88 0.94. IFN-r markedly decreased PI and FI in comparison with LPS or control. LPS and IFN-r treatment had synergistic effect since it increased higher PI and FI than individual agent treatment (Fig. 2).

4. PI and FI in macrophages after *G. scabra* extract treatment

Fig. 2 shows maximum PI in *G. scabra* extract treatment was 41.67 3.35 and maximum FI was 2.36 0.23. *G. scabra* treatment strongly decreased in comparison with control and pyrimethamine, indicating that *G. scabra* inhibits infection and proliferation of toxoplasma.

5. Electron microscopic observation

Macrophages in the presence or absence of *G. scabra* extract were appeared normal morphology (Plate 1 & 2). Cell membrane, endoplasmic reticulum and Golgi apparatus were apparent in macrophages and lysosomes were shown near Golgi apparatus. However, active lysosomes and mitochondria and remnants of *G. scabra* extract were shown in macrophages treated with *G. scabra* extract in comparison with control (Plate 1). After *T. gondii* infection, macrophages were incubated for 12 hr or 24 hr and observed under electron microscopy. After 12 hr incubation, it was observed 2 or 4 division forms of tachyzoites in cyst which were near mitochondria and increased vacuoles in infected cells (Plate 3). 4 or 8 tachyzoites were appeared in infected cells incubated for 24 hr and a few tachyzoites escaped from destroyed macrophages. Macrophages incubated for 24 hr had denser mitochondria located near cyst and more vacuoles than cells for 12 hr (Plate 5). After treated with *G. scabra* extract, macrophages were infected with *T. gondii* following 12 or 24 hr incubation. In macrophages incubated for 12 hrs, tachyzoites were deflated and their cell walls were irregular. A few destroyed tachyzoites were shown around activated mitochondria (Plate 4). After 24 hr incubation, vacuoles, endoplasmic reticulum and lysosomes increased and tachyzoites which were shown in macrophages (4 or 5) markedly decreased in comparison with them in control. This result indicates that *G. scabra* extract inhibits proliferation of *T. gondii* by destroying tachyzoites (Plate 6).

DISCUSSION

T. gondii is active in macrophagesdeficient O_2 or nitrogen[1,2,22]. Many studies reported nitric oxide is effective factor against intracellular protozoa[12,13,14,19]. Macrophages activated by IFN-r and LPS produced nitric oxide followed removal of intracellular toxoplasma[16]. We first examined whether *G. scabra* extract stimulates nitric oxide production in macrophages. *G. scabra* extract induced high nitric oxide production in comparision with LPS and IFN-r, indicating that *G. scabra* activates macrophages (Fig. 1). Soh *et al.* (1994)[16] demonstrated that *C. lacryma* decreased toxoplasma infection and growth. In this study, the *G. scabra* extract concentration of 0.1 mg/ml induced nitric oxide production. This results demonstrates the concentration of *G. scabra* extract is significantly low concentration in comparison with *C. lacryma* (30 mg/ml).

Since *G. scabra* activates macrophages, we confirmed therapeutic effect of *G. scabra* including inhibition of toxoplasma infection and proliferation in the next step. PI is an indicator of toxoplasma infection and FI is of toxoplasma proliferation. Macrophages were incubated with presence or absence of IFN-r or LPS or *G. scabra* extract, infected with *T. gondii* and stained with Giemsa dye. PI was measured as percentage of macrophage infected with *T. gondii*. FI was assessed as ratio of the mean number of *T. gondii* per 100 macrophages after 48 hr incubation to the

mean number of *T. gondii* per 100 macrophages at the beginning of the assay. Fig. 1 showed LPS and IFN-r markedly decreased PI and FI in comparision with control. *G. scabra* extract also decreased (Fig. 2). Specially, FI in *G. scabra* extract treatment is lower than in pyrimethamine, antitoxoplasmosis drug, treatment. This results concord with increased production of nitric oxide after *G. scabra* extract treatment, indicating that antitoxoplasmosis effect of *G. scabra* extract may be associated with nitric oxide production by activated macrophages. However, we do not know actual elements of *G. scabra* extract functioning antitoxoplasmosis. Purification of *G. scabra* extract is under investigation.

We also confirmed *G. scabra* extract inhibits proliferation of *T. gondii* by electron microscopy examination. After *G. scabra* extract treatment, macrophages were infected with *T. gondii* and subsequently incubated for 12 or 24 hrs. It was shown that macrophages are activated, tachyzoites are deflated and mitochondria and lysosomes in cell increased (Plate 3~6). This result indicates that *G. scabra* extract blocked growth of *T. gondii* by inhibiting protein or DNA metabolism of *T. gondii*. Recently, it was reported that infective *T. gondii* decreased phagosome formation and macrophagesmake phagosome acidify for phagosome activation[8]. In addition, vacuoles which is produced by phagocytosis of macrophages is associated with protein metabolism of host cells[15].

In conclusion, the present study provides *G. scabra* extract increases nitric oxide production and decreased PI and FI in macrophages suggesting that *G. scabra* extract has inhibitory effect of toxoplasmosis in macrophage. From this information, *G. scabra* extract may be used as a therapeutic agent against toxoplasma infection.

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