Efficacy of piperaceae plant extracts and microorganism-derived compounds: metacytofilin and kijimicin against *Toxoplasma gondii*

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トキソプラズマ・ゴンディに対する
コショウ科植物抽出物、微生物由来化合物
メタサイトフィリンおよびキジマイシンの効果

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Contents

Abbreviations and unit abbreviations

Abbreviations

A	ACI	- Anticoccidia index
B	BSA	- Bovine serum albumin
C	CCK-8	- Cell counting kit-8
	CNS	- central nervous system
D	DNA	- Deoxyribonucleic acid
	DMEM	- Dulbecco's modified Eagle's medium
	DMSO	- dimethyl sulfoxide
	dpi	- Days post-infection
E	E0	-pregnancy at day 0
F	FBS	- Fetal bovine serum
H	HFF	- Human foreskin fibroblast
	HIV	- human immunodeficiency virus
I	IFAT	- Immunofluorescent antibody test
	IC_{50}	- the half maximal inhibitory concentration
	Ig	- Immunoglobulin
	IP	-intraperitoneal route
M	MEM	- Minimum essential medium eagle
P	PBS	- Phosphate-buffered saline
	PCR	- polymerase chain reaction
	PLK	- type II low virulent strain of *T. gondii*
	PLK-GFP	- a green fluorescent protein expressing-PLK strain

	PO	-per oral route
	PVs	- parasitophorous vacuoles
R	RH-GFP	- a green fluorescent protein expressing-RH strain
	ROS	- reactive oxygen species
	RT	- room temperature
S	SI	- Selectivity index
V	Vls	- vacuole-like structure

Unit abbreviations

µl	-microliter	°C	-degree Celsius
ml	-milliliter	h	-hour
l	-lite	min	-minute
µM	-micromolar	%	-percentage
nM	-nanomolar		
pM	-picomolar		
mg/kg	-milligram/kilogram		
mg/ml	-milligram/milliliter		
µg/ml	-microgram/milliliter		
ng/ml	-nanogram/milliliter		

General introduction

1. Toxoplasmosis

1.1. Etiology and prevalence

Toxoplasmosis is one of the typical parasitic zoonosis caused by *Toxoplasma gondii* (*T. gondii*), an obligate intracellular parasite belongs to the phylum apicomplexan, subclass coccidian [Montoya and Liesenfeld, 2004]. Toxoplasmosis mainly impacts to the public health because of morbidity and mortality. Incidence of infection shows worldwide distribution as more than one-third of the human population are affected. *T. gondii* can infect most of the warm-blooded animals including the livestock, bird, wildlife, marine mammals and human, which have been accounted as the intermediate hosts, and its definitive hosts are Felidae family such as cat [Tenter *et al*., 2000; Dubey and Jones, 2008].

The prevalence of *T. gondii* which is determined by specific anti-*Toxoplasma* Immunoglobulin G (IgG) antibodies is widely distributed. The seroprevalence is lowest in the Far East approximately 1%. In European countries, the prevalence ranges between 10% to 60%, and in some parts of European and South American are more than 90% [Flegr *et al*., 2014]. The prevalence of *Toxoplasma* infection is affected by the several factors. Warm and humid climates, levels of food hygiene, behavior to consume raw food, life style and culture, contact with cats, lower levels of education, and occupations (i.e. farmer and housewife) are recognized as risk factors for the infection [Elsheikha, 2008; El Deeb *et al*., 2012; Daryani *et al*., 2014] .

1.2. Transmission and life cycle

T. gondii has complicated life cycle because it has an asexual stage in intermediate hosts and a sexual stage in definitive hosts [Black and Boothroyd, 2000]. Parasites possess several infective forms; tachyzoites, bradyzoites in tissue cysts and sporozoites in oocysts. Tachyzoites are crescent or oval shape, multiply rapidly, penetrate for entering to the host cells, and form a parasitophorous vacuole (PV) to protect the parasites from the host immune response. After the infection into host cells, the tachyzoites repeat multiplication until rupture of the infected cells. Then, tachyzoites distribute in the hosts via blood circulation and reach to the central nervous system (CNS), eye, and muscle. The tachyzoites convert to bradyzoites in order to escape from the host immune system. The bradyzoites grow slowly and persist inside the tissue cyst until decreasing of the host immunity. During the reactivation of the parasites under the suppression of host immune responses, the bradyzoites transform to the tachyzoites. In the definitive hosts, the parasites undergo a sexual replication to form male and female gametes in the gastrointestinal tract. After fertilization, immature oocysts are shed in cat feces and the oocysts become mature as sporulated oocysts which contain sporozoites [Montoya and Liesenfeld, 2004; Dubey, 2008; Hill and Dubey, 2016].

T. gondii mainly infects the intermediate hosts through horizontal transmission. Humans can be infected by consuming undercooked or raw meat contaminated with the tissue cysts or contacting the sporulated oocysts in the environment, soil, water, fruit and vegetable. [Robert-Gangneux and Dardé, 2012; Halonen and Weiss, 2013]. Congenital toxoplasmosis occurs via the horizontal transmission or the reactivation of the parasites in pregnant women. During the pregnancy, the tachyzoites vertically transmit through

the placenta and infect with the fetus. The other possible ways of the transmission are organ transplantation and blood transfusion [Tenter *et al*., 2000].

1.3. Clinical features and pathology

Clinical signs of toxoplasmosis usually are nonspecific. It is depend on host genetics, immunity, and duration of infection [Elsheikha, 2008]. Toxoplasmosis in immunocompetent hosts acquired after birth is asymptomatic or shows mild symptoms such as fever, cervical lymphadenopathy, headaches, and muscle pain. Because these clinical signs recover to normal, the treatments are not required for asymptomatic or mild symptoms. On the other hand, the infection in immunocompromised hosts and congenital infection is frequently fatal [Montoya, 2002; Singh, 2003; Montoya and Liesenfeld, 2004; Dubey and Jones, 2008].

The infection in immunocompromised hosts, either primary infection with *T. gondii* in acute stage or reactivation of latent bradyzoite cysts in chronic infection is detrimental to persons with lower immunity, resulting in severe clinical manifestations, such as pneumonia, encephalitis and hepatitis. The persons who undergo immunosuppression by drugs, organ transplantation or infected with Human immunodeficiency virus (HIV) always show life threatening [Robert-Gangneux and Dardé, 2012; Pittman and Knoll, 2015].

Congenital toxoplasmosis leads to a wide variety of manifestations. The infection during the first trimester pregnancy is more severe than the third trimester. During the early stage of pregnancy, the tachyzoites cross the placenta and then infect the fetus, resulting in abortion, still-birth, and abnormal development. Although newborn is

asymptomatic or develops physical abnormal, retinochloroiditis, CNS involvement, hydrocephalus, physical retardation, or abnormal of mental [Singh, 2016]. Additionally, ocular toxoplasmosis occurs through congenital infection or acquired infection after birth. The typical pathology of ocular toxoplasmosis is a focal necrotizing retinitis or retinochoroiditis including the white focal lesion. The lesions present due to the parasites invasion into the tissues, the immune response against the parasites or both. Clinical manifestations are also involved in the vision problems [Montoya and Liesenfeld, 2004; Singh, 2016].

1.4. Diagnosis

The clinical signs of toxoplasmosis are nonspecific, therefore the diagnosis must be the combination of biological, serological, or histological methods to achieve a definite diagnosis [Montoya, 2002]. Among the indirect methods, serologic tests based on the identifying of parasite-specific antibodies in patients are often used because the specific antibodies possess remarkable patterns after infection with *T. gondii*. Immunoglobulin A (IgA) and IgM are produced during first week after infection, and the levels of these antibodies reach the plateau within 1 month. Then, the levels of IgA, IgM dramatically decrease. While IgG levels increase within 1–2 weeks after the infection and normally persist for a lifetime [Montoya, 2002]. Several methods for detection of the antibodies have been used such as Sabin-Feldman dye test, ELISA, modified agglutination test (MAT), indirect fluorescent antibody test (IFAT). Sabin-Feldman dye test is gold standard for *Toxoplasma* diagnosis. It is specific and sensitive in human, but the live tachyzoites of *T. gondii* are needed for this test [Sabin and Feldman, 1948].

As direct-detection methods of *T. gondii*, a polymerase chain reaction (PCR) targeting the B1 gene of the *T. gondii* for the samples of cerebrospinal fluid or blood can be used [Dubey, 2008].

1.5. Treatment

The treatments should be considered in patients with toxoplasmosis, especially congenital toxoplasmosis, ocular toxoplasmosis, and cerebral toxoplasmosis. The first line regiment consists the combination of pyrimethamine and sulfadiazine plus folic acid to prevent bone marrow suppression from pyrimethamine [Montoya and Liesenfeld, 2004]. The combination of pyrimethamine and sulfadiazine shows synergistic effect, due to their effect on the folic acid biosynthesis. The sulfonamides inhibit dihydropteroate synthetase because sulfonamides are competitive antagonists of para-aminobenzoic acid. While pyrimethamine is an inhibitor of dihydrofolate reductase, which involves in thymidine synthesis that is one of the nucleotides in DNA. As a result, nuclear division is inhibited during the parasite replication [Zimmerman *et al*., 1987]. However, the activity of these drugs is limited in the tachyzoite stage in acute stage of infection. They cannot penetrate the bradyzoite membrane to act on the parasites. Moreover, the side effects are found as follow; allergy, kidney stones, hepatic or renal complications. The treatments need at least 1 year for congenital toxoplasmosis, and at least 6 weeks for *Toxoplasma* encephalitis and ocular toxoplasmosis. Therefore, the high rate of toxic effects is found in prolonged treatment procedures, resulting in discontinue of the therapy [Porter and Sande, 1992; McLeod *et al*., 2006].

Furthermore, cotrimoxazole, clindamycin, atovaquone, and spiramycin are the alternative drugs [Antczak *et al*., 2016]. Spiramycin (macrolide antibiotic) can be used for prophylactic to reduce the tachyzoites from mother to fetus due to macrolide cannot cross the placenta [Montoya and Remington, 2008].

2. Natural products

Natural products can be categorized from their original obtaining into four groups; (1) plant sources [Farnsworth *et al*., 1985], (2) marine organism sources (sponges, tunicates, cone shells, etc.) [Molinski *et al*., 2009; Kobayashi, 2016], (3) microorganism sources including minerals and (4) animal sources [Lam, 2007; Harvey, 2008].

Recently studies from 1981 to 2014, Newman and Cragg have reported that the new chemical entities from total 1,328 compounds, approximately 151 of which were generated from natural products, could be widely divided to antiparasitic drugs (e.g. artemisinin, ivermectin), antiviral drugs (e.g. laninamivir octanoate, interferon-alfa), antifungal drugs (e.g. anidulafungin, caspofungin acetate), antibacterial drugs (e.g. azithromycin, cetolozane, tazobactam). In the same time, nearly 50% of the new chemicals which approved for anticancer come from natural products; e.g. paclitaxel, epirubicin HCI, and cabazitaxel [Newman and Cragg, 2016]. In 2012, Berdy has been summarized that the bioactive compounds are derived from natural products according to their original source; approximately 3%, 7% and 47% of compounds obtained from animals, plants and microorganisms, respectively [Berdy, 2012]. From 2001 to 2010,

the largest group of microorganisms-derived compounds is produced from fungi and bacteria approximately 61% and 38.5% of the compounds, respectively [Berdy, 2012].

Discovery of the new compounds for treatment of toxoplasmosis is continued, and an importance sources for drug discovery are natural products. The plenty of plants, more than 20 plant species, had been studied on their anti-*Toxoplasma* activity for alternative toxoplasmosis treatments. Most of plant medicine are evaluated from their crude extracts from a family *Simaroubaceae*, *Ginkgoaceae*, *Fabaceae*, and *Simaroubaceae*, etc. [Sepulveda-Arias *et al*., 2014].

Not only plants, but also marine organisms, microorganisms and animals give the diversity of novel compounds for toxoplasmosis treatment. Marine-derived compounds, plakortolide and plakortolide G isolated from *Plakinastrella onkodes* sponge, have anti-*Toxoplasma* activity (IC_{50} of plakortolide and plakortolide G were 64 nM and 10 μM, respectively [Perry *et al*., 2001]. Muqubilin and sigmosceptrellin-B are norsesterterpene acid, which obtained from the Red Sea sponge *Diacarnus erythraeanus*, inhibit *T. gondii* at a concentration of 0.1 μM with nontoxic to host cells [El Sayed *et al*., 2001].

Animal toxin, L-amino acid oxidase isolated from snake venom, *Bothrops pirajai*. The toxin has a direct effect on the parasites because L-amino acid oxidase inhibits the parasite invasion than the intracellular parasite replication [Izidoro *et al*., 2011]. Neuwiedase, a metalloproteinase isolated from snake venom, *Bothrops neuwiedi*, has the similar results as the L-amino acid oxidase from *Bothrops pirajai*. The treatment RH strain of *T. gondii* before the parasite infection with neuwiedase is more effective than the treatment after the infection [Bastos *et al*., 2008].

The majority of bacteria-derived compounds are characterized from *Actinomycetaceae* family [Berdy, 2012], which can be classified into two groups; *Streptomyces* and non-*streptomyces* or also known as rare actinobacteria [Butler, 2004; Azman *et al*., 2015]. *Streptomyces* species represent the richest sources of antibiotics with bioactive secondary metabolites. The group of compounds obtained from *Streptomyces* spp. can be classified to polyene macrolides, macrolides, anthracyclines, polyether antibiotics, cyclopolylactones, most aminoglycosides, streptothricins, actinomycins and quinoxaline-peptides [Taddei *et al*., 2006; Berdy, 2012; Hasani *et al*., 2014]. Polyether ionophore such as monensin, salinomycin and inostamycin are produced from *Streptomyces* spp. Monensin and salinomycin are trendy use to control coccidian. The drugs have broad biological activity, antibacterial, antifungal, antiviral, anticancer, antiparasite including *T. gondii* as well [Kevin *et al*., 2009; Huczynski, 2012; Rutkowski and Brzezinski, 2013]. Although *Streptomyces* spp. can be used for characterizing the compounds, non-*streptomyces* also has a possibility for discovery of the novel compounds [Jose and Jebakumar, 2013]. Kijimicin, a polyether antibiotic, is produced from *Actinomadura* sp. MI215-NF3 (rare actinobacteria) [Takahashi *et al*., 1990]. Kijimicin has antibacterial and anticoccidia activities, and inhibits every step of HIV replication. The effects of kijimicin against chicken coccidian *Eimeria tenella* is more effective compared with monensin or salinomycin [Yamauchi *et al*., 1993].

Besides, fungi-derived compounds are also promising sources of novel bioactive compounds [Donzelli and Krasnoff, 2016]. Genus *Metarhizium* (Hypocreales: Clavicipitaceae) is known as the entomopathogenic fungal, which is currently used for alternative insecticide in many countries, Australia, Brazil, USA, and the Philippines

[Driver *et al*., 200]. The classification of *Metarhizium* is currently recognized as *M. anisopliae, M. flavoviride* or *M. album* [Driver *et al*., 200]. Numerous of bioactive compounds are produced from *Metarhizium* spp. Metacytofilin produced from *Metarhizium* sp. TA2759 has been proved for immunosuppressive effect [Iijima *et al*., 1992]. Furthermore, helvolic acid and its derivatives [Lee *et al*., 2008], and destruxins are obtained from *M. anisopliae* [Skrobek and Butt, 2005; Liu and Tzeng, 2012]. Metarhizin A, which is originally isolated from *M. flavoviride*, inhibits cytochrome c oxidase system, induces growth arrest of cells, and enhances cell death through the generation of reactive oxygen species (ROS) [Katou *et al*., 2014]. Nowadays, the study of fungi-derived compounds on anti-*Toxoplasma* activity is rare. Therefore, it should be more emphasized for searching new substance to treat toxoplasmosis.

In general, natural products are important quality sources of drug discovery for developing of human and animal health, particularly curing cancer and infection diseases, due to the diversity of chemical structures and biological properties found in it [Lam, 2007; Harvey, 2008; Dias *et al*., 2012; Newman and Cragg, 2016].

3. Aims of the present study

Toxoplasmosis causes severe life-threatening in immunocompromised patients and in congenital infection. The treatments are required for the severe clinical cases or congenital toxoplasmosis. The treatment regiments using the current drugs are limited because of the lower efficacy and the side effects. Therefore, searching the new substances is required for the novel treatment. The aim of my thesis is to evaluate the

crude extracts from piperaceae plants as plant source, kijimicin isolated from bacteria *Actinomadura* sp. and metacytofilin isolated from fungi *Metarhizium* spp. on anti-*Toxoplasma* activity in an *in vitro* and *in vivo*.

Chapter 1

Effects of extracts from Thai piperaceae plants against infection with *Toxoplasma gondii*

1-1. Introduction

Toxoplasma gondii, an obligate intracellular protozoan, causes toxoplasmosis. Infection with *T. gondii* threatens one-third of the global human population [Montoya and Liesenfeld, 2004]. *Toxoplasma* infections have nonspecific symptoms, but can be associated with several clinical syndromes and cause serious complications and severe life-threatening disease in congenitally infected and immunocompromised hosts. Ingestion of raw or undercooked meat containing *T. gondii* tissue cysts is the main route of infection for this parasite [Elsheikha, 2008; Weiss and Dubey, 2009]. Currently, sulfonamide drugs and pyrimethamine used in combination are the gold-standard medicines for treating toxoplasmosis [McLeod *et al*., 2006]. These drugs have a synergistic activity against tachyzoites, but have limited efficacy in eliminating *T. gondii* encysted bradyzoites [de Oliveira *et al*., 2009], and severe side effects and adverse drug reactions such as hematological reactions, embryopathies, bone marrow supression, hypersensitivity, and gastrointestinal disorders have been noted [Montoya and Remington, 2008; Furtado *et al*., 2011]. Therefore, the development of novel efficacious drugs with low toxicities is urgently needed.

Piperaceae plants comprise approximately 1,000 species of herbs, and are found in tropical areas of India, Southeast Asia and Africa [Scott *et al*., 2007]. Forty species of such plants have been identified in Thailand [Runglawan *et al*., 2012]. These plants are used as active ingredients in Thai traditional medicine and have many uses, such as ameliorating stress, improving digestion and nutrient absorption, and balancing general health; they also have antimalarial properties and are used for cancer treatment [Chaveerach *et al*., 2006; Suthanurak *et al*., 2010; Ruangnoo *et al*., 2012; Thiengsusuk *et al*., 2013]. Pharmacologically, the properties of piperaceae plants have been shown to be antibacterial, antioxidant, gastro protective, and anticancer [Rekha *et al*., 2014]. Furthermore, such plants have been shown to have anti-leishmanial activity [Misra *et al*., 2009] and anti-malarial activity [Al-Adhroey *et al*., 2010; Bagatela *et al*., 2013]. Because herbal medicines and natural herb extracts are widely used as alternative treatments for various parasitic diseases and some have been tested on *T. gondii in vitro* [Sepúlveda-Arias *et al*., 2014], I was interested in exploring whether piperaceae plants possess anti-*Toxoplasma* activity. Thus, the aim of this study was to evaluate the effects of ethanol extracts from Thai piperaceae plants (*P. betle*, *P. nigrum* and *P. sarmentosum*) on *T. gondii* infections *in vitro* and *in vivo*. My data indicate that of the three plants that I tested, *P. betle* extract has the potential to act as a medical plant for treating toxoplasmosis

1-2. Materials and Methods

Animals

Experiments were performed using female C57BL/6 mice (6–8 weeks old) obtained from Clea Japan, Inc. Six mice per cage were kept in the animal facility at the National Research Center for Protozoan Diseases (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan) under standard laboratory conditions with commercial food and water available *ad libitum*.

Parasites and cell cultures

The two strains of *T. gondii* used for the experiments, RH-GFP (a green fluorescent protein expressing-RH strain) [Nishikawa *et al*., 2003] and PLK-GFP [Nishikawa *et al*., 2008] (a PLK strain), were maintained in African green monkey kidney (Vero) cells cultured in minimum essential medium eagle (MEM, Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin, 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Parasites were propagated every three days. Parasites were purified by washing them in cold phosphate-buffered saline (PBS), and the final pellet was resuspended in cold PBS and passed three times through a 27-gauge needle syringe. Next, the parasites were filtered through a 5.0 µm pore filter (Millipore, Bedford, MA), washed twice with 10 ml of PBS, and centrifuged at 1,000 × *g* for 10 min. The parasites were refiltered and their numbers counted on a hemacytometer for each experiment. Human foreskin fibroblast (HFF) cells were maintained in Dulbecco's modified Eagle’s medium (DMEM, Sigma) supplemented with 10% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

Plant materials

Three types of piperaceae plants were used in this study. The fresh leaves of *Piper betle* L. 8,955 g and *P. sarmentosum* Roxb. 11,310 g were purchased from Don Wai Floating market in Nakhon Pathom province, and the 1,000 g dried seeds of *Piper nigrum* L., were purchased from herbal drug stores (Vejpong Pharmacy Co., Ltd) Thailand. All plant materials were identified by the Faculty of Pharmacy, Mahidol University. The plant serial numbers for *P. betle* L. are as follows: PBM05160, *P. nigrum* L.: PBM05159, *P. sarmentosum* Roxb.: PBM05161 (Fig. 1). The fresh leaves were cleaned with water. Only sound leaves were dried in a hot air oven at 70°C for 48 h, after which they were ground into small pieces. The extraction methods were modified from Choochote et al [Choochote *et al*., 2006]. Plant materials were extracted once with 97% ethanol (Sigma-Aldrich, St. Louis, MO, USA) at room temperature (RT) over a 3-day period. The solution was filtrated through sterile gauze and cotton, the filtrate was evaporated to dryness under reduced pressure at 40°C with a rotary evaporator (Rotavapor R-200/205, BÜCHI, Flawil, Switzerland), and then lyophilized using a Freeze Dry Vacuum System (Labconco, Kansas City, MO USA). The weights of the final crude extracts of *P. betle*, *P. sarmentosum* and *P. nigrum* were 43.42 g, 39.07 g and 51.18 g, respectively, and the yields of the extracts based on their dry weights [Maisuthisakul *et al*., 2007] were 3.05%, 3.97% and 3.93%, respectively. The final crude extracts were dissolved in dimethyl sulfoxide (DMSO) at 100 mg/ml and kept at -30°C.

Cytotoxicity tests

Cytotoxicity analysis of the three piperaceae crude ethanol plant extracts (*P. betle*, *P. nigrum*, *P. sarmentosum*) and sulfadiazine (Sigma-Aldrich) were conducted on HFF cells. Sulfadiazine was dissolved in 1-M NaOH (stock solution 200 mg/ml) according to the manufacturer's recommendations. Because 0.01 M NaOH did not inhibit HFF cell growth, I used 1 mg/ml sulfadiazine at the highest concentration in my study. HFF cells were plated at 100 µl/well in 96-well plates (cell suspensions 1×10^5 cells/ml in DMEM supplemented with 10% FBS), and then incubated at 37°C in a 5% CO_2 atmosphere for 48 h. Next, the cells were exposed to the piperaceae extracts at final concentrations of 1, 5, 10, 25, 50, 100 µg/ml, sulfadiazine (at 10 ng/ml to 1 mg/ml), and culture medium was used as a control. After 24 h, the cell viability was measured by adding cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Inc. Japan) to the cultures. The absorbance of the supernatant was measured at 450 nm using an MTP-120 micro plate reader (Corona Electric, Ibaraki, Japan). HFF cell viability (%) is expressed as [(the absorbance of cells treated with the extracts / (the absorbance of cells cultured with medium alone) × 100].

Indirect fluorescent antibody test (IFAT)

Vero cells, plated at 1 ml/well in 12-well plates (cell suspensions 1×10^5 cells/ml in MEM supplemented with 8% FBS), were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Coverslips were collected at 24 h after parasite inoculation, washed twice with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++), and then fixed with 3% paraformaldehyde in PBS++. After washing twice with PBS++, the cells were

permeabilized with 0.3% Triton X-100 in PBS++ for 5 min at RT. After washing, the coverslips were incubated with 3% bovine serum albumin (BSA) in PBS++ at RT for 30 min. To count the number of parasites in parasitophorous vacuoles (PVs), the coverslips were incubated with an anti-SAG1 monoclonal antibody (clone TP3; Advanced ImmunoChemical Inc., Long Beach, CA, USA) diluted 1:100 in 3% BSA in PBS++ for 1 h at RT. After washing three times with PBS++, the coverslips were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (Sigma) diluted 1:1,000 in 3% BSA in PBS++ for 1 h at RT, and then washed again with PBS++. Nuclear DNA was labelled with Hoechst 33342 (1:10,000 dilution, Thermo Fisher Scientific Inc., MA, USA) for 30 min. The coverslips were placed on a glass slide coated with Mowiol (Calbiochem, San Diego, CA, USA), and the slides were examined using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Tokyo, Japan).

Effects of piperaceae extracts on intracellular T. gondii in vitro

HFF cells, plated at 100 µl/well in 96-well plates (cell suspensions 1×10^5 cells/ml in DMEM supplemented with 10% FBS), were incubated at 37°C in a 5% CO_2 atmosphere for 48 h. To examine the effects of this treatment on the intracellular parasites, RH-GFP and PLK-GFP (5×10^4 tachyzoites per well) were added to the wells for 4 h and the extracellular parasites were washed away. Then, the piperaceae extracts at final concentrations of 1, 5, 10, 25, 50, and 100 µg/ml (100 µl/well of media) were added for 72 h. Sulfadiazine (1 mg/ml, Sigma) and control media were used as positive and negative controls, respectively. The fluorescence intensity of RH-GFP and PLK-GFP were measured using a microplate reader (SH-900, Corona Electric Co., Ltd, Ibaraki,

Japan). The correlation coefficient between the fluorescence intensity of GFP and the number of parasites (a two-fold serial dilution ranging from 1,000,000 to 7812.5 parasites) was calculated using the Pearson correlation coefficient and a positive correlation was confirmed (RH-GFP: r = 0.992, PLK-GFP: r = 0.969). The growth inhibition of RH-GFP and PLK-GFP (%) was expressed as follows: [(average fluorescence intensity of GFP with medium alone) − (the fluorescence intensity of GFP treated with either of the extracts or sulfadiazne) / (average fluorescence intensity of GFP with medium alone)] × 100. The half maximal inhibitory concentration (IC_{50}) values of the plant extracts and sulfadiazine on *T. gondii* were calculated based on three independent experiments performed together by GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Additionally, to measure *T. gondii* replication in vero cells, the PV sizes for RH-GFP expressed as a percentage (%) were determined by counting the number of parasites per PV (a total of 25 randomly selected vacuoles) at 24 h after infection (post-treatment with the extract as described above) based on the SAG1 signal measured by IFAT, as described above.

Effects of piperaceae extracts on extracellular T. gondii in vitro

RH-GFP tachyzoites (2×10^5) were pretreated with either of the three piperaceae extracts (25 μg/ml), sulfadiazine (1 mg/ml), or MEM alone (1 ml/tube) for 1 h at 37°C. Then, the pretreated parasites were added to vero cells at 1 ml/well in a 12-well plate (parasites per host cell ratio = 2:1). At 2–3 h post-infection, the extracellular parasites were washed away and MEM supplemented with 8% FBS was added. To determine the percentage inhibition of the *T. gondii* tachyzoites, the infection rates at 24 h post-

infection were calculated by IFAT as follows: [(number of SAG1-positive vero cells) / (100 randomly selected vero cells)] × 100. The percentage inhibition of the tachyzoites was expressed as follows: [(average infection rate after treatment with medium alone) − (infection rate after treatment with either of the extracts) / (average infection rate after treatment with the medium alone)] × 100.

Effects of the piperaceae extracts on T. gondii infections in vivo

Mice were intraperitoneally inoculated with *T. gondii* (PLK strain, 1×10^3 tachyzoites/mouse). At 24 h post-infection, the mice were intraperitoneally injected with the *P. betle* extract at either 25 (n = 6), 100 (n = 12), and 400 mg/kg/24 h (n = 12), or PBS (n = 12) for 7 days. To further evaluate the anti-*Toxoplasma* activity of the *P. betle* extract, the mice infected with 1,000 and 100,000 PLK strain *T. gondii* tachyzoites (n = 6 per group) were treated with 400 mg/kg of *P. betle* extract or PBS via the intraperitoneal route, or 400 mg/l sulfadiazine via the drinking water as a standard treatment [Saeij *et al*., 2005] for 7 days. The mice were observed daily for 30 days post-infection. Daily observations such as body weight, morbidity, mortality and clinical signs were noted, as were the clinical scores. Most of the signs recorded were assessed by the criteria used in other infection studies with protozoan parasites [Hermes *et al*., 2008; Carroll *et al*., 2010]. The scores varied from 0 (no signs) to 10 (all signs). The clinical signs recorded included hunching, piloerection, worm-seeking behavior, ptosis, sunken eyes, ataxia, latency of movement, deficient evacuation and touch reflexes, and lying on belly. The brains from the surviving mice

were collected to determine the parasite burden by quantitative PCR, as described previously [Tanaka *et al*., 2013].

Statistical analysis

GraphPad Prism 5 software (GraphPad Software Inc.) was used. Data represent the mean ± SD. Statistical analyses were performed using Student's *t*-test, a one-way or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons. Survival curves were generated by the Kaplan–Meier method, and statistical comparisons were made using the log-rank method. The levels of statistical significance are shown as asterisks or letters and defined in each figure legend together with the name of the statistical test that was used. A *p* value of $P < 0.05$ was considered statistically significant.

1-3. Results

Cytotoxicity of piperaceae extracts and sulfadiazine

To analyze the toxicity of each piperaceae extract on HFF cells *in vitro*, I examined cell proliferation using a CCK-8 cell counting kit. When exposed to 100 μg/ml of *P*. *betle* extract for 24 h, the cell proliferation rate was 66.19%, while the proliferation rates of HHF cells treated with either 1, 5, 10, 25 or 50 μg/ml of *P*. *betle* extract were more than 100% (Fig. 2A). Therefore, the safe concentration of *P*. *betle* extract was considered to be < 50 μg/ml in this study. However, the proliferation rates of the HFF cells treated with 100 μg/ml

of *P. nigrum* or *P. sarmentosum* extract were more than 100% (Fig. 2A), indicating that these extracts had lower cytotoxicity than that of the *P. betle* extract. The IC_{50} value of the *P. betle* extract against HFF cell growth was 180.2 μg/ml (Fig. 2B). There was no obvious cytotoxic effect of sulfadiazine on the HFF cells, even at the highest concentration (1 mg/ml) (Fig. 3C).

Effects of the piperaceae extracts and sulfadiazine on T. gondii growth in vitro

To analyze the anti-*Toxoplasma* effects of each piperaceae extract *in vitro*, I examined the fluorescence intensity of RH-GFP. At 72 h post-treatment, the *P. betle* extract inhibited RH-GFP growth at concentrations 25 and 50 μg/ml showing 63.4 ± 16.9% and 96.8 ± 7.2% inhibition, respectively (Fig. 4A). However, the *P. nigrum* and *P. sarmentosum* extracts had no effects on RH-GFP growth (Fig. 4C). The IC_{50} of the *P. betle* extract on RH-GFP was 23.2 μg/ml, while those of the *P. sarmentosum* and *P. nigrum* extracts were > 100 μg/ml. Furthermore, the *P. betle* extract also inhibited the growth of PLK-GFP (IC_{50}: 21.4 μg/ml) (Fig. 4B). Although sulfadiazine inhibited the growth of *T. gondii* (IC_{50} on RH-GFP: 99.4 μg/ml, IC_{50} on PLK-GFP: 22.3 μg/ml), the GFP signal was still observed, even at the highest concentration of sulfadiazine (1 mg/ml) (Fig. 3).

Effects of the piperaceae extracts on intracellular and extracellular T. gondii in vitro.

To examine the effect of each piperaceae extract on extracellular *T. gondii*, purified extracellular parasites pretreated with 25 μg/ml of either extract were used to infect vero cells (Fig. 5A). Pretreatment with the *P. betle* extract resulted in 100%

inhibition of the parasite infection while pretreatment with extracts from *P. sarmentosum* or *P. nigrum* resulted in 94.7 ± 5.3% and 63.2 ± 5.3% inhibition, respectively (Fig. 5B). Treatment with the *P. betle* extract resulted in no GFP signal from the RH-GFP parasites (Fig. 5A), suggesting that destruction of the parasite cell membrane and release of GFP from the cytosol had occurred. All extracts were more effective at parasite inhibition than 1 mg/ml of sulfadiazine (29.8 ± 10.9%) (Fig. 5B).

To test the effect of each piperaceae extract on intracellular *T. gondii*, RH-GFP-infected vero cells were treated with 25 µg/ml of either extract or 1 mg/ml of sulfadiazine (Fig. 6A). Only two parasites per PV were found in the cells treated with the *P. betle* extract at 24 h post-infection (Fig. 6B). Additionally, the GFP signal from the parasites was lower in cells treated with the *P. betle* extract (Fig. 6A), indicating the anti-*Toxoplasma* effect of this extract. Sulfadiazine or *P. sarmentosum* treatment caused only slight inhibition of parasite replication (Fig. 6B).

Effects of the piperaceae extracts on T. gondii infections in vivo.

Because the *P. betle* extract had anti-*T. gondii* activity *in vitro*, I evaluated the effects of the *P. betle* extract on *T. gondii in vivo* (Fig. 7A). Although higher clinical scores were seen in the PBS-injected mice from 10 to 13 days post infection (dpi), treatment with *P. betle* extract at 400 and 100 mg/kg reduced the clinical signs from 10 to 13 dpi and from 12 to 13 dpi, respectively (Fig. 7A). Furthermore, treating the infected mice with 400 and 100 mg/kg of the *P. betle* extract resulted in 100% and 83.3% mouse survival, respectively (Fig. 7B). However, the survival rates of the mice treated with 25 mg/kg of *P. betle* extract or PBS was 33.3% (Fig. 7B). This result indicates that treatment

with 400 and 100 mg/kg of *P. betle* extract ameliorated toxoplasmosis in mice during the acute infection.

To evaluate further the anti-*Toxoplasma* activity of *P. betle* extract, I performed additional experiments to compare the extract-treated group of mice with the sulfadiazine-treated mouse group (Fig. 8). In the case of infection with 1,000 tachyzoites, the survival rate of the *P. betle*–treated mouse group was 100%, while one mouse died at 14 dpi in sulfadiazine-treated group and all mice died within 13 dpi in the PBS-injected group (Fig. 8A). The clinical scores of the *P. betle*- and sulfadiazine-treated mice against infection with 1,000 *T. gondii* were significantly lower than those of the PBS-injected mice from 9 to 12 dpi and 11 to 12 dpi, respectively (Fig. 8A). There was no significant difference in the parasite numbers in the brains of the surviving mice between the *P. betle*-treated and sulfadiazine-treated groups (Fig. 8A). Furthermore, all mice in the *P. betle*-treated group survived the infection with 100,000 tachyzoites, but two mice died at 12 and 15 dpi in the sulfadiazine-treated group, and all mice died within 10 dpi in the PBS-injected group (Fig. 8B). Treatment of the infected mice with *P. betle* or sulfadiazine decreased the clinical signs during the acute phase of the infection from 6 to 9 dpi (Fig. 8B). The clinical score from 7 to 9 dpi and the number of parasites in the brains of the surviving mice in the sulfadiazine-treated group were significantly lower than those of *P. betle*-treated group (Fig. 8B). Altogether, treatment with the *P. betle* extract controlled acute toxoplasmosis in the mice, although some parasites were detectable in their brains.

1-4. Discussion

Toxoplasmosis is one of the most important and challenging diseases in public health. To control *T. gondii* infection and the toxoplasmosis caused by it, herbal medicine and natural herb extracts are of growing interest. There are many traditional herbal medicines with antimicrobial and antihelminthic properties, and some have anti-*Toxoplasma* activities such as *Curcuma longa* [Al-Zanbagi, 2009], *Eurycoma longifolia* Jack [Kavitha *et al*., 2012; Khanam *et al.*, 2015], and *Myristica fragrans* Houtt [Pillai *et al*., 2012]. Moreover, the anti-*T. gondii* activity of *Artemisia annua* L. [Nagamune *et al.*, 2007; El Zawawy, 2008; de Oliveira *et al*., 2009], *Dichroa febrifuga* [Jain *et al*., 2015], herbal extracts from South Korea (*Sophora flavescens, Sinomenium acutum, Pulsatilla koreana, Ulmus macrocarpa* and *Torilis japonica*) [Youn *et al*., 2003], and *Eurycoma longifolia* [Kavitha *et al*., 2012] have been reported. However, piperaceae extracts have not been tested for their potential anti-*Toxoplasma* effects even though they have many pharmacological properties including activities against fungi [Ali *et al*., 2010], insects [Scott *et al*., 2007], protozoa [Al-Adhroey *et al*., 2010; Thiengsusuk *et al*., 2013; Fazal *et al*., 2014], helminthes [Philip *et al*., 1984; Atjanasuppat *et al*., 2009] and cancer cells [Ruangnoo *et al*., 2012]. Only extracts of *P. nigrum* have been shown to possess *in vivo* activity, with a reported *T. gondii* growth inhibition of 78.3% and 86.3% with treatment doses 100 and 200 mg/kg/day, respectively [Al-Zanbagi, 2009], but there is no *in vitro* information for this extract. Herein, I evaluated the effects of ethanol extracts of *P. betle, P. nigrum* and *P. sarmentosum* from Thailand on *T. gondii* growth *in vitro* and *in vivo*.

Importantly, the *P. betle* extract had anti-*Toxoplasma* activity both *in vitro* and *in vivo*. In the *in vitro* tests, 25 µg/ml of the *P. betle* extract eradicated extracellular and intracellular parasites. The selective activity of the treatment was considered through the selectivity index (SI). Both *P. betle* extract and sulfadiazine were calculated as the ratio between cytotoxic IC_{50} values and parasitic IC_{50} values [Koch *et al.*, 2005]. The IC_{50} values of the *P. betle* extract for HFF cells, RH-GFP and PLK-GFP were 180.2 µg/ml, 23.2 µg/ml and 21.4 µg/ml, respectively. Therefore, each SI for RH-GFP and PLK-GFP was 7.77 and 8.42, respectively. Because there was no obvious cytotoxic effect of sulfadiazine on the HFF cells, even at the highest concentration (1 mg/ml), the SI was not able to be used in my study. In a previous study, de Oliveira et al [de Oliveira *et al*., 2009] reported that the IC_{50} value of sulfadiazine on *T. gondii* (RH strain) in HFF cells was 70 µg/ml, and the viability of HFF cells in the presence of 200 µg/ml of sulfadiazine decreased by 28% (no SI value was reported). Schoondermark-van de Ven et al [Schoondermark-van de Ven *et al*., 1995] reported that the IC_{50} of sulfadiazine against the growth of *T. gondii* in human epithelial type 2 (HEp-2) cells could not be determined because the drug was toxic to HEp-2 cells at concentrations above 1,000 µg/ml. Thus, in comparison with sulfadiazine, *P. betle* extract should be effective at controlling the growth of *T. gondii in vitro*.

Furthermore, treatment of *T. gondii*-infected mice with the *P. betle* extract increased the survival rates of the mice, particularly at the highest concentration (400 mg/kg) of extract that I used. When compared with sulfadiazine treatment, treatment with *P. betle* extract (400 mg/kg) produced better mouse survival rates, although *T. gondii* DNA was still detectable in the mouse brains. Thus, treatment

with *P. betle* extract was effective at controling acute toxoplasmosis in mice. In this study, I did not test the toxicity of *P. betle* extract *in vivo*, but a previous study showed that an ethanol extract of *P. betle* leaves (after oral administration of 1,500 mg/kg/day for 40 consecutive days) produced no significant side-effects to cross-bred male albino rats in terms of clinical signs, food intake, percent weight gain and serum parameters [Arambewela *et al*., 2005], indicating that my regimen should be reliable.

Extracts of *P. nigrum* and *P. sarmentosum* were also tested *in vitro* in my study. *P. nigrum* and *P. sarmentosum* each showed an inhibitory effect against extracellular *T. gondii*, but not against intracellular parasites. My results indicate that *P. nigrum* and *P. sarmentosum* extracts affected the extracellular parasites, while the *P. betle* extract was effective against extracellular and intracellular parasites alike. The main chemical classes found in *P. betle* leaves are alkaloids, terpenes, anthraquinones, flavonoids, tannins, saponins and steroids [Al-Adhroey *et al.*, 2010]; more specifically, chavibetol, chavibetol acetate, allylpyrocatechol diacetate, eugenol, safrole, quercetin, a-Pinene, f-pinene, u-limonene, and saprobe [Al-Adhroey *et al*., 2010; Bhalerao *et al*., 2013; Dwivedi and Tripathi, 2014; Rekha *et al*., 2014]. Furthermore, phytochemical compounds such as alkaloids and flavonoids have antiplasmodial activities [Al-Adhroey *et al*., 2010]. Therefore, any one or a combination of these compounds might affect *T. gondii* growth. In a future study, I will test the effects of the phytochemical compounds that were found in *P. betle* extract for their anti-*Toxoplasma* activities.

1-5. Summary

In the present study, three piperaceae plants; *P. betle*, *P. nigrum*, and *P. sarmentusum* have been tested anti-*Toxoplasma* activity in an *in vitro* and *in vivo*. I found that piperaceae plants had anti-*Toxoplasma* activity particularly *P. betle* crude extract. *P. betle* had little toxicity to HFF cells and effectively inhibited intracellular *T. gondii* growth (RH and PLK strains). The potential of *P. betle*, *P. nigrum*, and *P. sarmentusum* crude extracts on extracellular parasites was more than sulfadiazine. *P. betle* crude extract eradicated the extracellular parasites to infect the host cells because of the destruction of parasite cell membrane. *In vivo*, *P. betle* crude extract could improve the clinical signs of infected mice in acute phase of infection and increased the survival rate of infected mice. Because *P. betle* crude extract has anti-*Toxoplama* activity, the any one or a combination phytochemicals that contain in *P. betle* leaves might affect *T. gondii* growth.

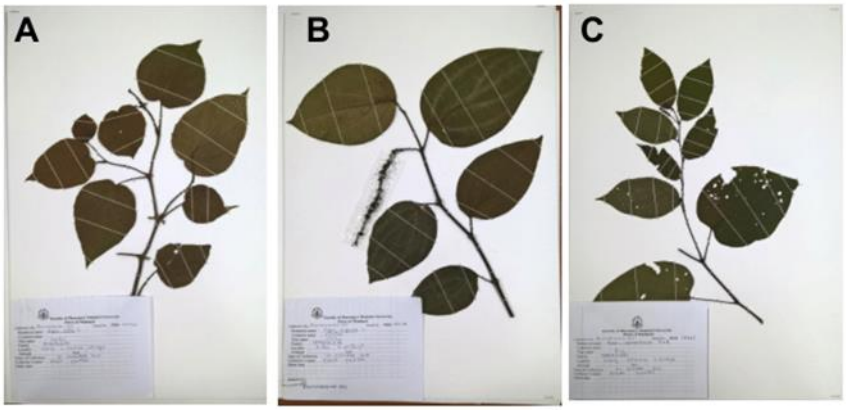

Fig 1. Plant materials.

(A) *Piper betle* L., (B) *P. nigrum* L., and (C) *P. sarmentosum* Roxb. were identified and transferred to the herbarium by the Faculty of Pharmacy, Mahidol University, 447 Sri-Ayuthaya Road. Rajathevi Bankok 10400, Thailand. The serial numbers given were as follows: *P. betle* L.: PBM05160, *P. nigrum* L.: PBM05159, *P. sarmentosum* Roxb.: PBM05161.

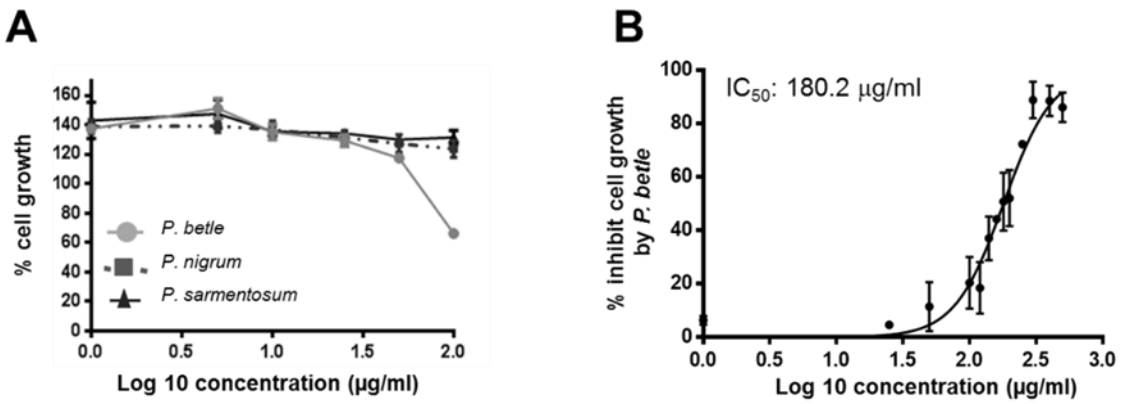

Fig 2. Cytotoxicity of the piperaceae extracts. (A) Cytotoxicity testing of HFF cells after treatment with either one of three piperaceae extracts (*P*. *betle*, *P*. *nigrum*, or *P*. *sarmentosum*) at concentrations of 0 to 100 µg/ml for 24 h. Data represent the mean values ± SD for three independent experiments. (B) Inhibition HFF cell growth by the *P*. *betle* extract. HFF cells were exposed to *P*. *betle* extract at concentrations 0 to 500 µg/ml. Data represent the mean values ± SD for three independent experiments. The IC_{50} value of the *P*. *betle* extract on HFF cells was calculated based on three independent experiments performed together.

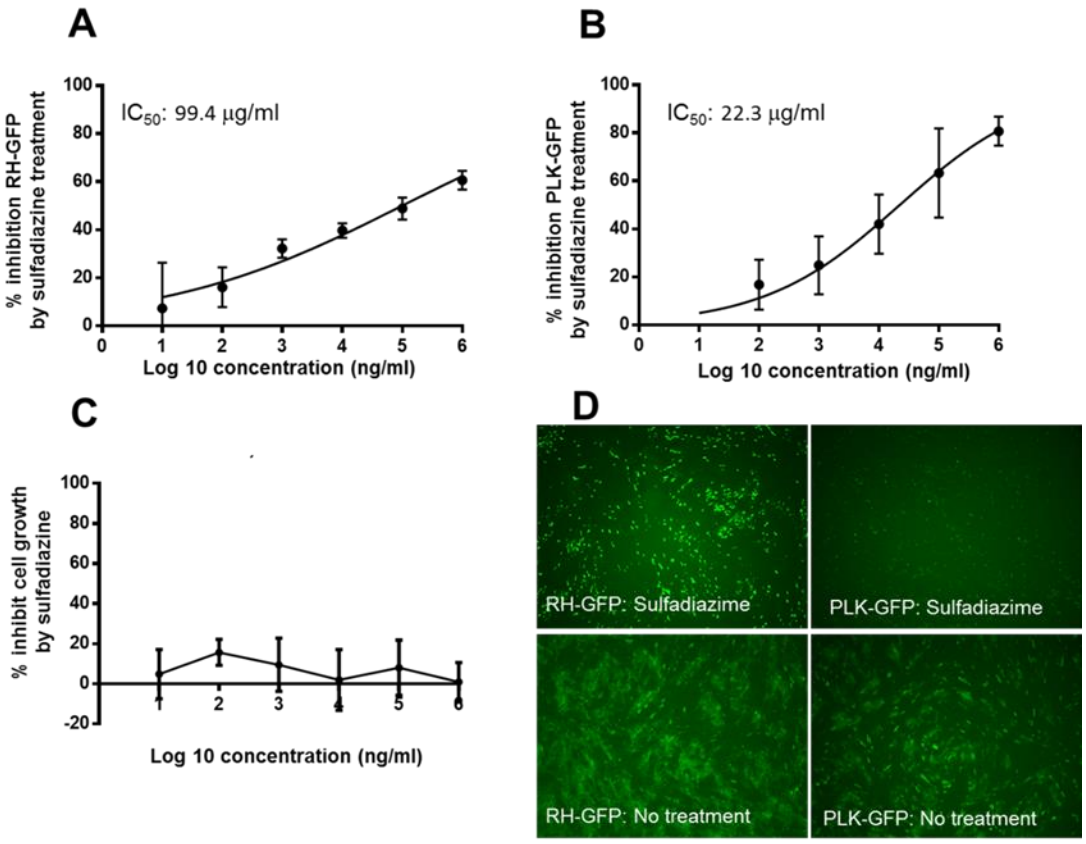

Fig 3. Anti-*Toxoplasma* activity of sulfadiazine on RH-GFP and PLK-GFP, and the cytotoxicity of sulfadiazine to HFF cells. Anti-*Toxoplasma* activity of sulfadiazine against RH-GFP (A) and PLK-GFP parasites (B). The RH-GFP and PLK-GFP-infected HFF cells were treated with sulfadiazine for 72 h at concentrations of 10 ng/ml to 1 mg/ml and the IC_{50} values were calculated for RH-GFP and PLK-GFP. Data represent the mean values ± SD for three independent experiments. (C) Inhibition of HFF cell growth by sulfadiazine. HFF cells were exposed to sulfadiazine at the highest concentration of 1 mg/ml. Data represent the mean values ± SD (n = 3). (D) Representative images of *T. gondii* RH-GFP- and PLK GFP-infected HFF cells treated with sulfadiazine (1 mg/ml) and culture medium alone (no treatment).

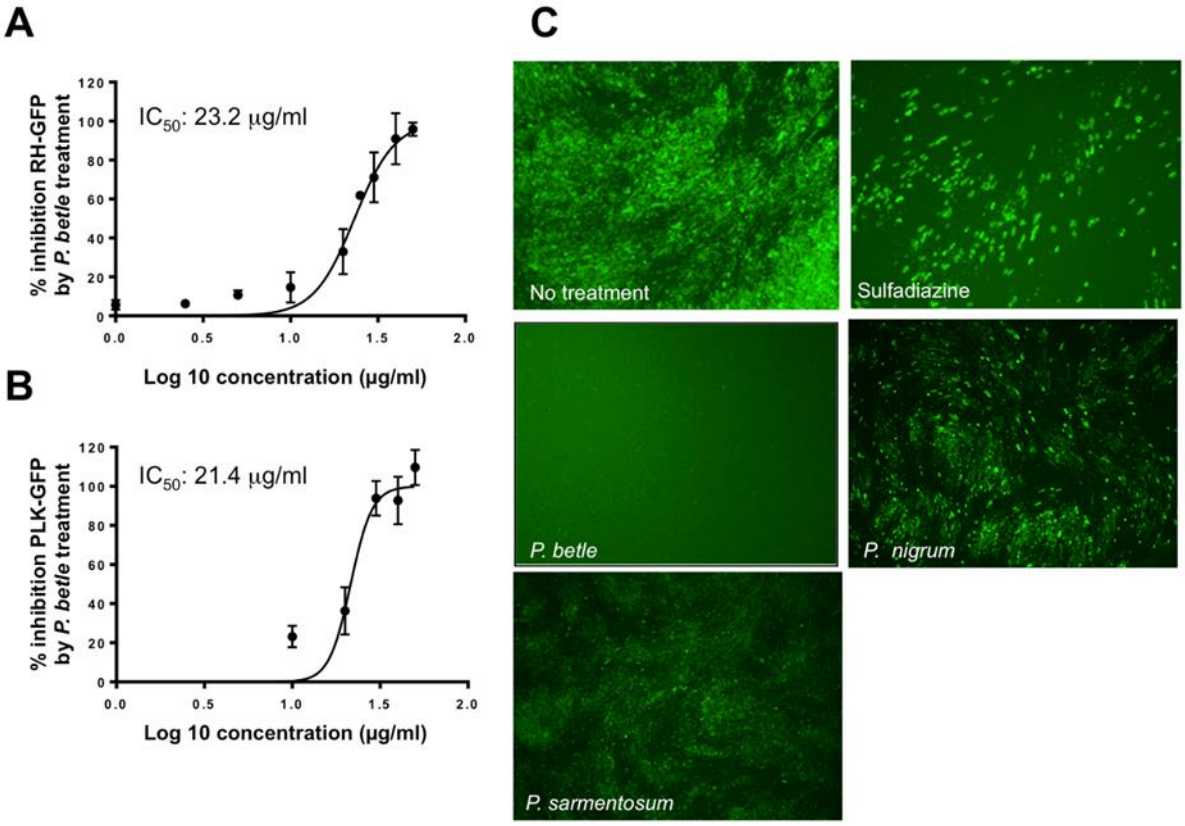

Fig 4. Anti-*Toxoplasma* activity of *P. betle* extract on RH-GFP and PLK-GFP. Anti-*Toxoplasma* activity of the *P. betle* extract on intracellular parasites RH-GFP (A) and PLK-GFP (B). The RH-GFP and PLK-GFP-infected HFF cells were treated with the *P. betle* extract for 72 h at different concentrations from 0 to 50 μg/ml. Data represent the mean values ± SD for three independent experiments. The IC_{50} values of the *P. betle* extract on RH-GFP and PLK-GFP were 23.2 μg/ml and 21.4 μg/ml, respectively. (C) Representative images of *T. gondii* RH-GFP-infected HFF cells treated with sulfadiazine (1 mg/ml), or either *P. betle* (50 μg/ml), *P. nigrum* (50 μg/ml) or *P. sarmentosum* (50 μg/ml) extract.

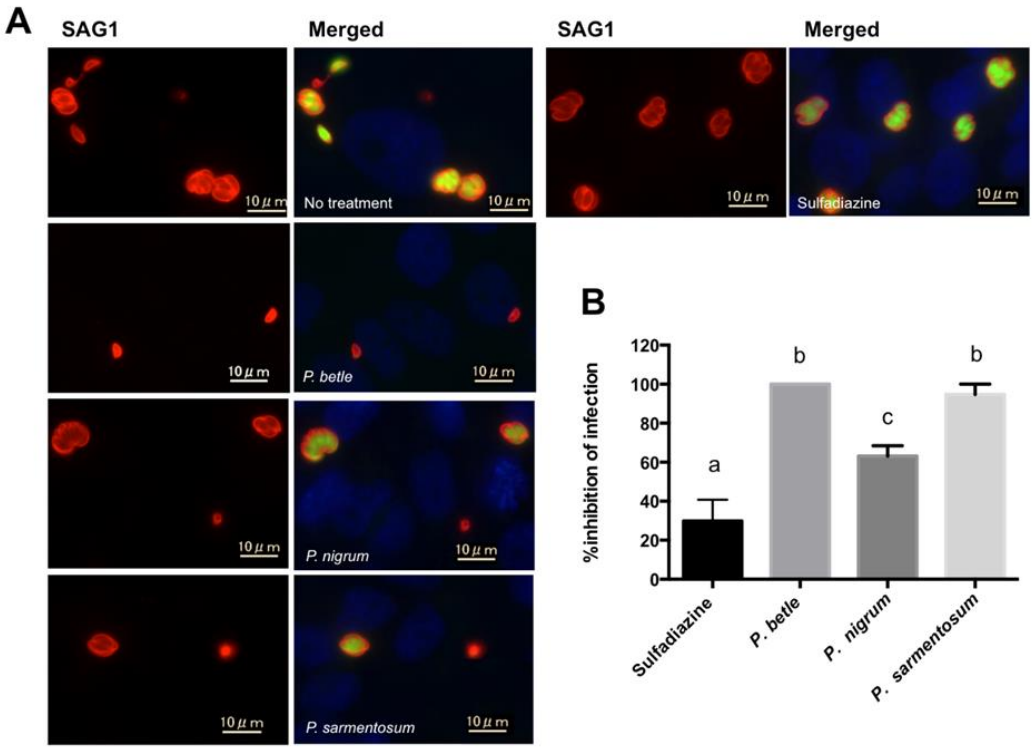

Fig 5. Effects of the three piperaceae extracts at 25 μg/ml and sulfadiazine at 1 mg/ml on extracellular *T. gondii*. The RH-GFP line, pre-treated with either of the extracts (*P. betle*, *P. nigrum* or *P. sarmentosum*) or sulfadiazine for 1 h, was then used to infect vero cells. After 24 h, the infected cells were analyzed by IFAT to measure the infection rates. (A) Representative images of *T. gondii* RH-GFP-infected vero cells. The cells were treated with either *P. betle* (25 μg/ml), *P. nigrum* (25 μg/ml), *P. sarmentosum* (25 μg/ml) extract or sulfadiazine (1 mg/ml). SAG1, red; GFP, green; nucleus, blue. (B) The % inhibition of infection for RH-GFP was measured by counting the number of SAG1-positive vero cells per 100 vero cells. Each bar represents the mean ± SD of three wells per group. The results represent two independent experiments. The different letters above the data bars in the graphs indicate statistically significant differences as determined by a one-way ANOVA plus Tukey–Kramer post-hoc analysis ($P < 0.05$).

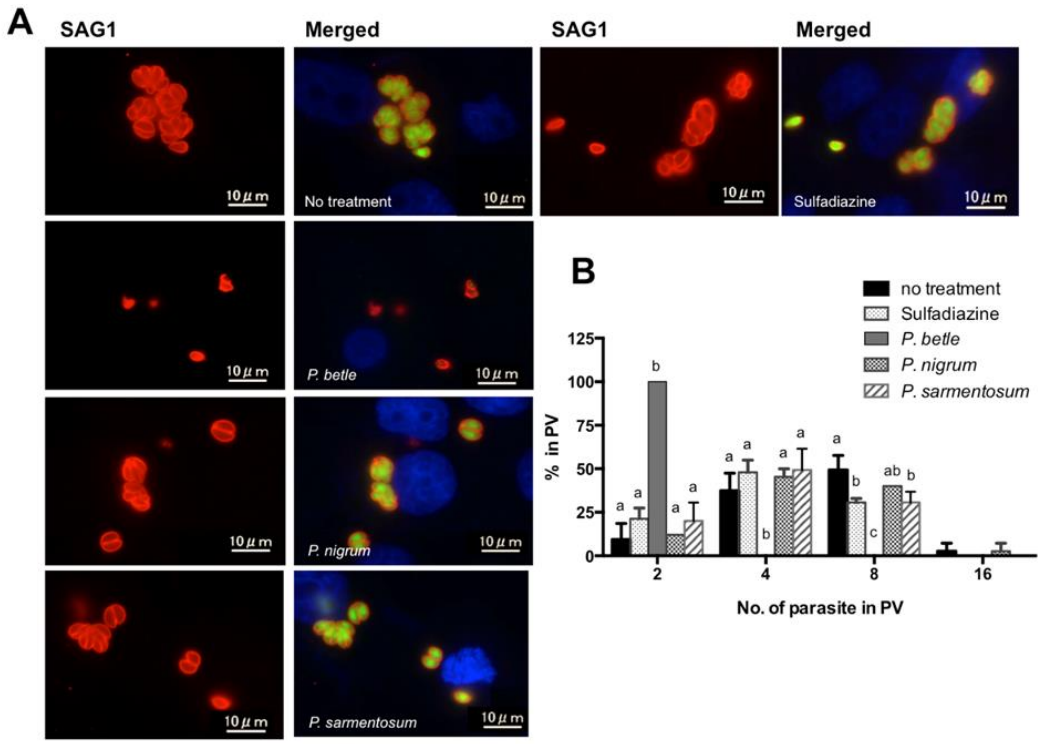

Fig 6. Effects of the three piperaceae extracts at 25 μg/ml and sulfadiazine at 1 mg/ml on intracellular *T. gondii*. The RH-GFP-infected vero cells were treated with either of the three piperaceae extracts or sulfadiazine for 72 h, and were then analyzed by IFAT to measure the parasitophorous vacuole (PV) sizes. (A) Representative images of *T. gondii* RH-GFP-infected vero cells. The cells were treated with either *P. betle* (25 μg/ml), *P. nigrum* (25 μg/ml), *P. sarmentosum* (25 μg/ml) extract, or sulfadiazine (1 mg/ml). SAG1, red; GFP, green; nucleus, blue. (B) The number of parasites in PVs was measured by counting the number of SAG1-positive parasites per PV. Each bar represents the mean ± SD of three wells per group. Results represent two independent experiments. The different letters above the data bars in the graphs indicate statistically significant differences in the number of parasites in PVs as determined by two-way ANOVA plus Tukey–Kramer post-hoc analysis ($P < 0.05$)

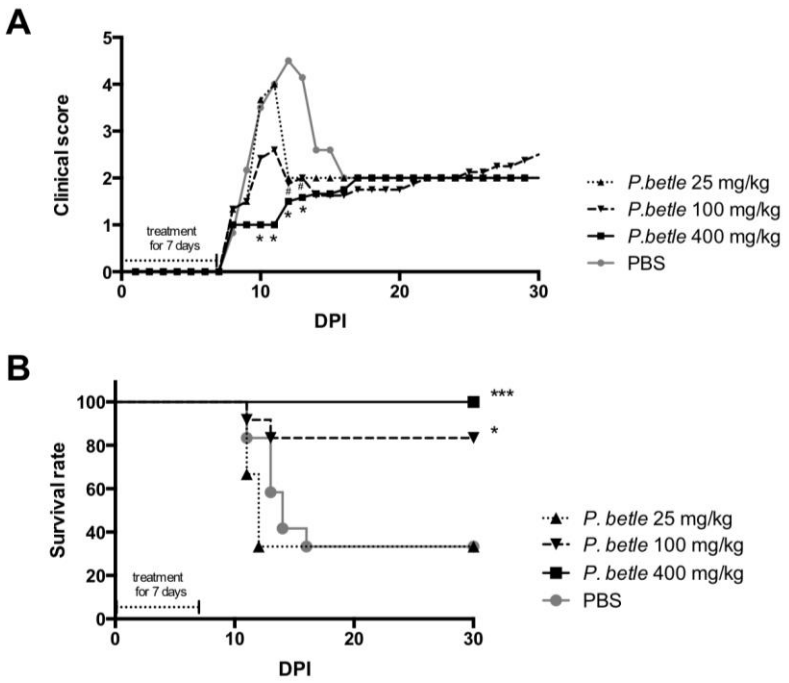

Fig 7. Clinical scores and survival of *T. gondii*-infected mice treated with *P. betle* extract. Mice were intraperitoneally administrated with *P. betle* extracts at 25, 100 and 400 mg/kg/day or PBS from 1 to 7 days post-infection with 10^3 PLK tachyzoites per mouse. Clinical scores (A) and survival (B) were monitored for 30 days post-infection in the mice. The clinical scores represent the mean total values for all mice used in this study. Data represent the mean values of all the mice used in two independent experiments performed together (*P. betle* extract at 100 and 400 mg/kg/day, PBS, $n = 6 + 6$; *P. betle* extract at 25 mg/kg/day, $n = 6$). The clinical scores were analyzed by two-way ANOVA plus Tukey–Kramer post-hoc analysis at the time points indicated (*Difference between PBS and *P. betle* extract at 400 mg/kg/day, $P < 0.05$; # Difference between PBS and *P. betle* extract at 100 mg/kg/day, $P < 0.05$). Survival curves were generated with the Kaplan–Meier method. According to the log-rank test, the differences between the PBS and *P. betle* extracts were significant (*Difference between PBS and *P. betle* extract at

100 mg/kg/day, $P < 0.05$; *** Difference between PBS and *P. betle* extract at 400 mg/kg/day, $P < 0.001$).

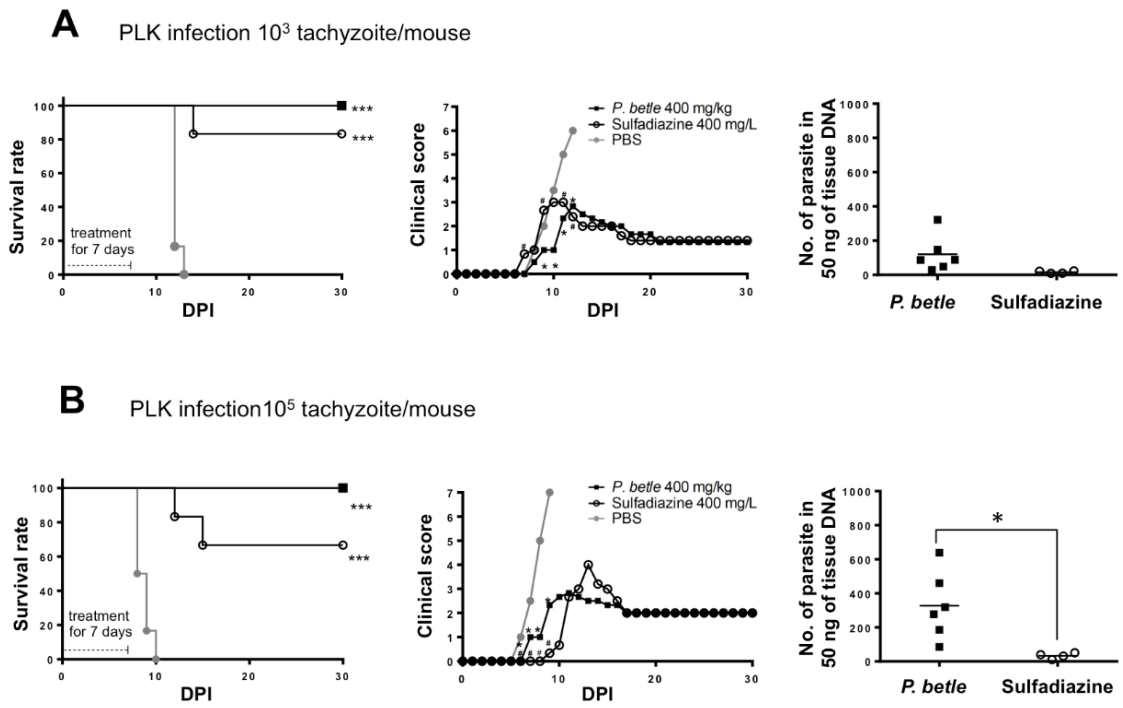

Fig 8. Survival, clinical score and parasite burden of mice infected with 1,000 and 100,000 *T. gondii* tachyzoites under treatment with *P. betle* extract or sulfadiazine. Mice infected with 1,000 (A) and 100,000 tachyzoites (B) of the *T. gondii* PLK strain (*n* = 6) were treated with 400 mg/kg *P. betle* extract, PBS via the intraperitoneal route, or 400 mg/l sulfadiazine via drinking water as a standard treatment from 1 to 7 days post-infection (dpi). Survival and clinical scores were monitored for 30 dpi and the brains from the surviving mice were collected to determine the parasite burden at 30 dpi. Survival curves were generated by the Kaplan–Meier method. According to the log-rank test, the differences between the PBS and *P. betle* extracts were significant (***Difference between PBS and *P. betle* extract or sulfadiazine treatment, $P < 0.001$). The clinical scores represent the mean total values for all the mice used in this study. Data represent the mean values of all the mice. The clinical scores were analyzed by two-way ANOVA plus Tukey–Kramer post-hoc analysis at the time points indicated (*Difference between PBS and *P. betle* extract, $P < 0.05$; # Difference between PBS and

sulfadiazine, $P < 0.05$). The number of parasites in 50 ng of tissue DNA per individual (symbols) and the mean levels (horizontal lines) are indicated. A significant difference between the two groups was observed by a student's *t*-test. (*Difference between treatment with sulfadiazine and *P. betle* extract, $P < 0.05$)

Chapter 2

In vitro and *in vivo* activities of polyether ionophore, kijimicin, against *Toxoplasma gondii*

2-1. Introduction

Toxoplasma gondii is an obligate intracellular parasites, and infects almost of the warm-blooded animals including humans, livestock, pets, wildlife, and marine mammals [Dubey and Jones, 2008]. Toxoplasmosis has worldwide distribution affecting approximately one-third of the world's human population [Tenter *et al*., 2000]. Human infection occurs via mainly horizontal transmission either by ingestion of infectious oocysts from the contaminated food and water, or ingestion of tissue cysts in undercooked meat. The infection during pregnancy causing congenital toxoplasmosis occurred by tachyzoites pass through placenta and infect the fetus [Tenter *et al*., 2000; Luder *et al*., 2001; Montoya and Liesenfeld, 2004; Sibley *et al*., 2009]. The infection is usually asymptomatic in most immunocompetent patients [Dubey and Jones, 2008]. In contrast, the infection causes severe life-threatening in immunocompromised individuals. Moreover, in congenital toxoplasmosis, the parasites in chronic stage may reactivate regarding to low immunity and reinfect to several tissues, resulting in severe manifestations presenting as chorioretinitis, encephalitis, pneumonitis [Montoya and Liesenfeld, 2004].

Toxoplasmosis treatment is required in case of immunocompromised individuals, congenital toxoplasmosis, or the severe clinical signs. Currently, the chemotherapeutic regimens using pyrimethamine combined with a sulfonamide drug plus folinic acid are used [Petersen *et al.*, 2003]. The combination of drugs has a curative effect against acute toxoplasmosis because of synergistic effects on disturbing folic acid biosynthesis. However, the drugs have toxic side effects which associated with bone marrow suppression and allergic reactions.

To decrease the side effects from sulfonamide, pyrimethamine is combined as alternative drugs such as lincosamides, clindamycin, azithromycin, or spiramycin [Antczak *et al*., 2016]. Azithromycin and spiramycin are alternative drugs for congenital toxoplasmosis treatment. Azithromycin inhibits the protein synthesis by acting on the 70S unit of ribosomes, and reduces the vertical transmission of the parasites through the placenta [Costa *et al*., 2009]. Because spiramycin can penetrate to intracellular and affect to intracellular pathogens, it inhibits the protein synthesis by its irreversible binding to the 50s ribosomal subunit. Spiramycin is also used to prevent *T. gondii* transmission from mother to the fetus [Montoya and Remington, 2008; Tamaru *et al*., 2011; Singh, 2016]. Clindamycin, which interrupts protein synthesis, is used for treatment of *Toxoplasma* retinochoroiditis by combining with dexamethasone [Harrell and Carvounis, 2014]. Clindamycin is well absorbed from the gastrointestinal tract but poorly distributes to the cerebrospinal fluid. Although this therapy is effective, it may cause the side effects [Antczak *et al*., 2016]. The chemicals for treatment of toxoplasmosis have the limitation on efficacy and side effects, discovery of new substances for treatment of toxoplasmosis will be challenging.

In recent year, studies of polyether ionophores produced by *Streptomyces* spp. have an impact because polyether antibiotics have a broad spectrums of bioactivity ranging from antibacterial, antifungal, antiparasitic, antiviral, and anticancer [Huczynski, 2012; Rutkowski and Brzezinski, 2013]. Polyether antibiotics such as monensin, salinomycin and lasalocid have anti-*Toxoplasma* activity [Melton and Sheffield, 1975; Kirkbride, 1992; Ricketts and Pfefferkorn, 1993]. Several compounds of polyether are widely used in veterinary field for control and prevention of coccidiosis in livestock [Couzinet *et al*., 1994; Chapman *et al*. 2010]

Kijimicin is a natural compound belonged to a polyether group. It was found from bacterium *Actinomadura* sp. MI215-NF3 taken from soil sample collected at Bunkyo-ku, Tokyo, Japan [Takahashi *et al*., 1990]. Kijimicin inhibits the growth of gram-positive bacteria and chicken coccidian *Eimeria tenella*. The effect of kijimicin is higher than monensin and salinomycin based on an anticoccidia index (ACI); ACI = (mean weight gain + survival rate) - (oocyst scores + lesion scores)]. The ACI of kijimicin is 156.6 while ACI of monensin and salinomycin are 144.1 and 127.1, respectively [Takahashi *et al*., 1990]. Moreover, kijimicin inhibits HIV replication in both infected lymphoid lineage H9 cells and mononuclear phagocyte lineage U937 cells, and shows activity in an *in vitro* assays of both acute and chronic infections [Nakamura *et al*., 1992; Yamauchi *et al*., 1993]. Although kijimicin has anticoccidia activity, the effect of kijimicin on anti-*Toxoplasma* activity is unknown. Here I evaluated the activity of kijimicin against *T. gondii* in an *in vitro* and *in vivo*. The obtained results showed that kijimicin inhibited intracellular parasite growth and invasion of the extracellular parasites into the host cells, and protected mice against *T. gondii* infection.

2-2. Materials and methods

Ethics statement

This study was performed in strict accordance with recommendations from the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 28-113, 29-45). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering.

Animals

In vivo study were conducted on the female BALB/c mice aged 6–8 weeks old obtained from Clea Japan, Inc. Two independent experiments were performed together using at least 6 mice per group (n = 6 + 6), mice were kept in the animal facility at the National Research Center for Protozoan Diseases (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan) under standard laboratory conditions with commercial food and water available *ad libitum*.

Host cell and parasites cultures

African green monkey kidney (Vero) cells and human foreskin fibroblast (HFF) cells were used in an *in vitro* studies. Vero cells were cultured in minimum essential medium eagle (MEM, Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin, 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. HFF cells were maintained in Dulbecco's modified

Eagle's medium (DMEM, Sigma) supplemented with 10% FBS, 100 U/ml penicillin, and 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. *T. gondii* tachyzoites of the type I RH strain expressing green fluorescent protein (RH-GFP) [Nishikawa *et al*., 2003] and the type II strain, PLK strain expressing GFP (PLK-GFP) [Nishikawa *et al*., 2008] were used in this study. Parasites were maintained and passaged through vero and HFF cells. Parasites were harvested by washing infected cells in cold phosphate-buffered saline (PBS), scraping infected cells followed by suspending in media and syringed through a 27-gauge needle syringe. Then parasites were filtrated through a 5.0 μm pore filter (Millipore, Bedford, MA), washed twice with 10 ml of media, and centrifuged at 1,000 × *g* for 10 min. The parasites were refiltrated and their numbers counted on a hemacytometer for each experiment.

Compounds

Kijimicin was kindly provided from Institute of Microbial Chemistry (BIKAKEN). Azithromycin, spiramycin, and clindamycin were obtained from Tokyo Chemical Industry Co., Ltd. Kijimicin, azithromycin, and spiramycin were prepared in dimethyl sulfoxide (DMSO) at 10 mM. Clindamycin was stocked in distilled water at 10 mM. Sulfadiazine and monensin were obtained from Sigma-Aldrich, prepared in ethanol at 200 mg/ml and 1 mM, respectively. All compounds were kept at -30°C until use.

Cytotoxicity assays

Cell suspension of HFF cells (1×10^4 cells/100 μl/well) were seeded in 96 well-plates. The cell proliferation was characterized after treatment of the monolayer

HFF cells with kijimicin ranging from 100 nM to 300 μM, monensin ranging from 1 nM to 10 μM, azithromycin ranging from 1 μM to 1,000 μM, clindamycin ranging from 50 μM to 1,000 μM, spiramycin ranging from 1 μM to 400 μM, and medium alone as a control group. At 72 h after the treatment, Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc. Japan) was added for the determination of cell viability. The absorbance values of each treated-well was measured at 450 nm using an MTP-120 micro plate reader (Corona Electric, Ibaraki, Japan). The percentage inhibition of HFF cell growth was determined as follows; [(average absorbance of cells treated without treatment) − (the absorbance of cells treated with either of compound) / (average absorbance of cells treated without treatment)] ×100.

Effects of compounds on inhibition intracellular T. gondii growth

HFF cell suspensions (1×10^4 cells/100 μl/well) were seeded in 96-well plates. After 48 h, the purified tachyzoites (5×10^4 cells/100 μl/well) were added to 80–90% confluent HFF cells. At 4 h after the infection, the extracellular parasites were washed away. The infected HFF cells were then treated with kijimicin ranging from 1 nM to 300 nM, monensin ranging from 1 pM to 5 nM, azithromycin ranging from 1 μM to 100 μM, clindamycin ranging from 1 nM to 500 nM, spiramycin ranging from 1 μM to 1,000 μM, or media alone. At 72 h after the treatment, the growth of *T. gondii* was measured by the fluorescence intensity using a microplate reader (SH-900, Corona Electric Co., Ltd, Ibaraki, Japan) as described previously [Leesombun *et al*., 2016]. The growth inhibition of RH-GFP (%) was determined as follows; [(average fluorescence intensity of GFP with

medium alone) − (the fluorescence intensity of GFP treated with either of compound) / (average fluorescence intensity of GFP with medium alone)] × 100.

Effects of Kijimicin and monensin on extracellular T. gondii in vitro

RH-GFP tachyzoites (2×10^5) were pretreated with kijimicin at 1 pM to 2,000 pM, and monensin at 1 pM to 4,000 pM or MEM alone (1 ml/tube) for 1 h at 37°C. Then, the pretreated parasites were added to vero cells at 1 ml/well in a 12-well plate (parasites per host cell ratio = 2:1). At 3 h post-infection, the extracellular parasites were washed away and MEM supplemented with 8% FBS was added. To detect RH-GFP in vero cells, the cells were washed twice with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++), and then fixed with 3% paraformaldehyde in PBS++. After washing twice with PBS++, Nuclear DNA was labelled with Hoechst 33342 (1:10,000 dilution, Thermo Fisher Scientific Inc., MA, USA) for 30 min. The coverslips were placed on a glass slide coated with Mowiol (Calbiochem, San Diego, CA, USA), and the slides were examined using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Tokyo, Japan). To determine the percentage inhibition of the *T. gondii* tachyzoites, the infection rates at 24 h post-infection were calculated as follows: [(number of GFP-positive vero cells) / (100 randomly selected vero cells)] × 100. The percentage inhibition of the tachyzoites was expressed as follows: [(average infection rate after treatment with medium alone) − (infection rate after treatment with kijimicin or monensin) / (average infection rate after treatment with the medium alone)] × 100.

Electron Microscopy

HFF cells were plated in 12 well-plate (1×10^5 cells/1 ml/well). After 24 h, HFF cells were infected with *T. gondii* RH-GFP (2×10^5 cells/well). After 4 h, the cells were washed with PBS once, fresh media contained kijimicin 50 nM and only media were added. After 24 h of treatment, the infected cells were washed with PBS twice, fixed in 3% Glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature. Then, the fixing solution was aspirated and washed with 0.1 M phosphate buffer 3 times. The infected cells were scraped and suspended with 0.1 M phosphate buffer 500 µl, followed by post fixation in 1% osmium tetroxide. Cells were dehydrated in an ethanol series, infiltrated with LR white resin and polymerized overnight at 60°C. Thin sections (90 nm) were placed on copper grids (stained with 4% uranyl acetate and lead) and observed with HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

Effects of kijimicin on T. gondii infections in vivo

Mice were intraperitoneally inoculated with *T. gondii* (PLK-GFP strain, 5×10^3 tachyzoites/mouse). At 24 h post-infection, the mice were intraperitoneally injected with kijimicin at 10 mg/kg/day (n = 12), 3 mg/kg/day (n = 6), and PBS (n = 12) for 7 days. Daily observations such as body weight, morbidity, mortality and clinical signs were noted for 30 dpi, as were the clinical scores. The scores were given according to hunching, piloerection, worm-seeking behavior, ptosis, sunken eyes, ataxia, latency of movement, gait, flaccid, touch reflexes, and lying on belly that varied from 0 to 10 [Hermes *et al*., 2008; Carroll *et al*., 2010].

Statistical analysis

All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc.) Data were shown as representative of mean ± SD. Statistical analyses, the group comparisons were performed using a two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group comparisons. The Kaplan–Meier method, and statistical comparisons between the curves using the log-rank method were used for survival curves. A *P* value of < 0.05 was considered statistically significant are shown as asterisks or symbol and defined in each figure legend together with the name of the statistical test.

2-3. Results

Effects of compounds on T. gondii growth and HFF cell proliferation

IC_{50} values of kijimicin, monensin, azithromycin, clindamycin and spiramycin on inhibition of HFF cell proliferation were 46.5 μM, 1.4 μM, 72.2 μM, 370.5 μM and, 61.8 μM respectively. Because the IC_{50} values of kijimicin, monensin, azithromycin, clindamycin and, spiramycin were 45.6 nM, 1.3 nM, 28.2 μM, 238.5 nM and 85.1, the selectivity index (SI) of these compounds were 1019.7, 1076.9, 2.6, 1553.5 and 0.7, respectively. Although the SI values of kijimicin, monensin and clindamycin were over than 1,000, treatment with monensin showed inhibitory effects against intracellular *T. gondii* at lower concentration (IC_{50}: 1.3 nM) and caused about 20% loss of HFF viability. Although cytotoxicity of sulfadiazine could not be calculated, the effects against *T. gondii* were lower than other compounds.

Furthermore, kijimicin affected the extracellular parasites, resulting in the inhibition of the parasite invasion. The IC_{50} and the SI of kijimicin on the extracellular *T. gondii* were 216.6 pM and 214,681.4, respectively (Fig. 9A). These results suggested that kijimicin directly affected the tachyzoites because the IC_{50} against extracellular parasites was lower than that against intracellular parasites. Similarly, monensin was more effective on extracellular parasites than intracellular parasites (IC_{50} on extracellular parasites: 513.1 pM, IC_{50} on intracellular parasites: 1.3 nM), suggesting that monensin might have the same effects to the extracellular tachyzoites as kijimicin by directly affecting to the tachyzoites.

To determine the ultrastructure of the parasites treated with kijimicin, morphological analyses by electron microscopy were performed (Fig. 10). In the presence of kijimicin, tachyzoite appeared cell swollen and found the multiple intracellular vacuole-like structures compared with the no treatment group (longitudinal section). The changing of ultrastructure of *T. gondii* may deficit normal function of the parasites and undergo parasite lysis.

Effects of kijimicin on T. gondii infection in vivo

The mice infected with *T. gondii* PLK-GFP strain and were treated with kijimicin at 3 and 10 mg/kg/day (Fig. 11). One mouse from 10 mg/kg/day-treated mice died at 25 dpi, and two mice from 3 mg/kg/day-treated animals died at 17 and 19 dpi (Fig. 11A). The all PBS-treated mice died within 18 dpi (Fig. 11A). Although the clinical symptoms were observed from 7 dpi, the treatment of infected mice with kijimicin decreased the clinical signs from 7 to 17 dpi (Fig. 11B).

2-4. Discussion

Toxoplasmosis causes a serious global health problems [Montoya and Liesenfeld, 2004]. The severe symptoms are found particular in immunosuppressed individuals. Treatment of toxoplasmosis in pregnant women is required to prevent the parasite transplacental transmission or to treat an infected fetus. Moreover, children with congenital infection should be also considered [Montoya and Liesenfeld, 2004; Petersen, 2007]. However, the treatment with the current drugs show some side effects and affect to only tachyzoite stage. Therefore, discovery of the alternative drugs are important [Antczak *et al*., 2016].

Polyether ionophores are important group which contains the biological active substances. Polyether ionophores are generally used for control of coccidiosis in ruminant. They have broad-spectrum activities in gram-positive bacteria [Rutkowski and Brzezinski, 2013]. These compounds have ability to form complexes with cation and transport them across cell membranes. In consequence, the whole process leads to change in the osmotic pressure inside the cell, causing cell swelling and death [Dubey and Jones, 2008; Rutkowski and Brzezinski, 2013]. It has been known that some polyether antibiotics such as monensin, salinomycin and lasalocid, have anti-*Toxoplasma* activity **[**Melton and Sheffield, 1975; Ricketts and Pfefferkorn, 1993; Gupta, *et al*., 2009]. Kijimicin has wide range of pharmacological properties such as anticoccidia, antibacterial and antiviral activities. Its efficacy on coccidia is higher than monensin and salinomycin. Here, I proved that kijimicin might be one of the good candidates to control *Toxoplasma* infection.

In my study, kijimicin inhibited *T. gondii* infection both *in vitro* and *in vivo*. The

IC_{50} of kijimicin, monensin and clindamycin were lower values compared with other compounds used in this study. Because the SI of kijimicin, monensin and clindamycin on intracellular parasites were more than 1,000, these compounds have high selective toxicity to the *T. gondii*. Furthermore, IC_{50} of kijimicin on extracellular parasites was 216.6 pM, indicating that kijimicin is more effective on extracellular parasites than intracellular parasites as monensin did. Although the mechanism of kijimicin against *T. gondii* is still unclear, my results of transmission electron microscopy showed that the swollen parasites and several vacuole-like structures were observed in the kijimicin-treated parasites (Fig. 10). These appearance of ultrastructure changings are also observed in monensin-treated *T. gondii* [Lavine and Arrizabalaga, 2012]. Based on pharmacology properties of polyether ionophore, the inhibition effects on parasites might be involved in disturbed balance of membrane permeability, due to the Na^+ influx as monensin that causes osmotic swelling and may alter the cell cycle [Couzinet *et al*., 2000; Lavine and Arrizabalaga, 2011]. Monensin affects to the free sporozoites or merozoites of the avian *Eimeria* spp. by blinding with sodium ion because the ionophore-cation complex is transported across the membrane and the cation is released [Mollenhauer *et al*., 1990]. Therefore, high intracellular of sodium concentration and change of the intracellular osmolality may lead swelling of the sporozoites [Smith *et al*., 1981; Smith and Galloway, 1983]. Lavine and Arrizabalaga (2011) show that the effect of monensin is dependent on the function of a mitochondrial homologue of the MutS DNA damage repair enzyme (TgMSH-1). Furthermore, monensin induces cell cycle arrest and autophagy for triggering the parasite death [Lavine and Arrizabalaga, 2011]. Additionally, monensin induces disruption of mitochondrial function caused by the

induction of an oxidative stress, suggesting that the parasite redox biology as a viable target for the development of drugs against *Toxoplasma* and related pathogenic parasites [Charvat and Arrizabalaga, 2016]. Thus, anti-*Toxoplasma* activity of kijimicin may be dependent on similar mechanism of monensin.

Administration of polyether ionophores should be considered because of its toxicity. Proliferation of fibroblast L929 cells treated with monensin at 1 μM at 48 h significantly decreased compared with control fibroblasts [Souza *et al*., 2005]. In my study, the IC_{50} of monensin on HFF cell proliferation was 1.4 μM. In an *in vivo* study, monensin treatment at 13.5 mg/kg for five consecutive days via gastric gavage is toxic to goat [Deljou *et al*., 2014]. On the other hand, the IC_{50} of kijimicin on HFF cell growth was 46.5 μM, indicating lower toxicity than monensin. Treatment with kijimicin controlled the *T. gondii* infection *in vivo* because the survival rate of the infected mice that were treated with kijimicin at 3 and 10 mg/kg/day were significantly higher than that of the PBS-treated animals. Previous report has been indicated that an acute toxicities (LD_{50}) of kijimicin in mice are 56 mg/kg for intraperitoneal injection and 180 mg/kg for oral administration [Takahashi *et al*., 1990]. Additionally, I confirmed death of mice intraperitoneally treated with kijimicin at 30 mg/kg. Thus, the administration dose should be considered to use kijimicin *in vivo*.

2-5. Summary

Instead of the old regimens, novel compounds with more effective and lower side effects for treating not only toxoplasmosis but also the other infection diseases such as

malaria are required. Therefore, discovery of natural product-derived compounds have been now focused on. In this study, I found the anti-*Toxoplasma* effects of kijimicin. Although cytotoxicity of kjimicin was relatively lower than that of monensin, safety of kjimicin administration should be studied more detail. The lethal effect of polyether ionophores is in part due to production of reactive oxygen species that are produced by mitochondria, and autophagy. Thus, further exploring compounds that induce an oxidative stress, interfere with parasite redox biology, induction of autophagy will be a better strategy for therapeutic development against parasitic infections including *T. gondii*.

Table 1. *In vitro*, the effects of compounds on anti-*Toxoplasma* activity and cytotoxicity

Drugs	IC_{50}		Selectivity index
	HFF cells	*T. gondii* RH-GFP	
Kijimicin	46.5 µM	45.6 nM	1019.7
Monensin	1.4 µM	1.3 nM	1076.9
Azithromycin	72.2 µM	28.2 µM	2.6
Clindamycin	370.5 µM	238.5 nM	1553.5
Spiramycin	61.8 µM	85.1 µM	0.7
Sulfadiazine	–	397.2 µM *	N.D.

-IC_{50} = the half maximal inhibitory concentration

-***** [Leesombun *et al*., 2016]

-N.D. = not determined

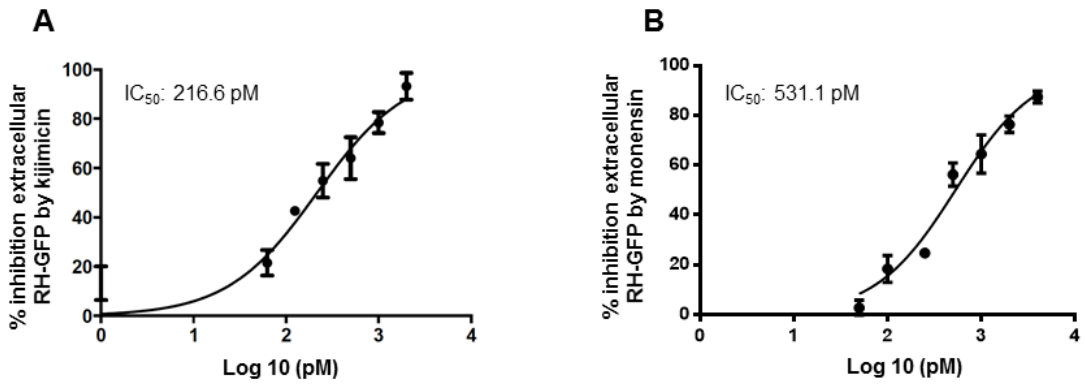

Fig 9. Effects of kijimicin and monensin on extracellular parasites. *T. gondii* RH-GFP was pretreated with several concentrations of kijimicin ranging from 1 pM to 2,000 pM and monensin ranging from 1 pM to 4,000 pM, or media as control in triplicate for 1 h, then allowed to infect vero cells. After 24 h, the infected cells were fixed, analyzed by measuring the infection rates and calculated the percentage inhibition of parasite infection. (A) Data represent the mean values ± SD for three independent experiments, IC_{50} of kijimicin on extracellular parasites was 216.6 pM. (B) Data represent the mean values ± SD for three independent experiments, IC_{50} of monensin on extracellular parasites was 513.1 pM.

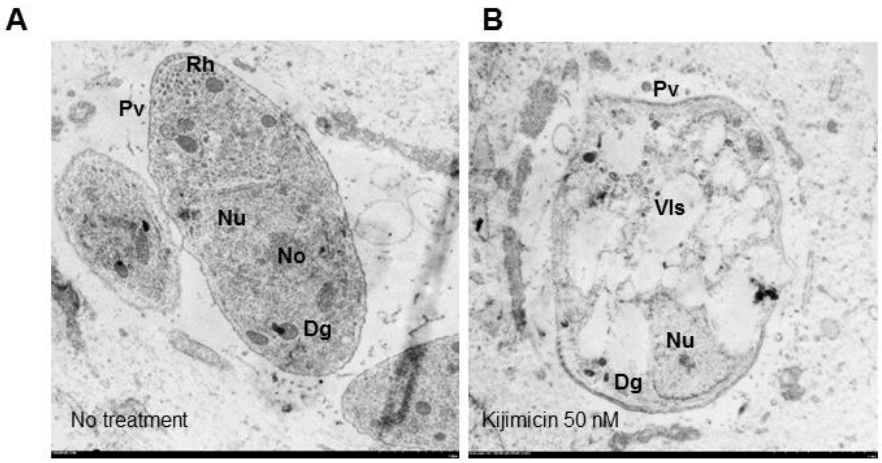

Fig 10. Tranmission electron microscographs of intracellular parasites. The infected vero cells were treated with media (no treatment) (A) or kijimicin 50 nM (B) for 24 h, then the infected cells were fixed for observed the ultrastructure by tranmission electron microscope. Pv, parasitophorous vacuole; Rh, rhoptries; Nu,nucleus; No,nucleous; Dg, dense granule; Vls. Vacuole-like structure.

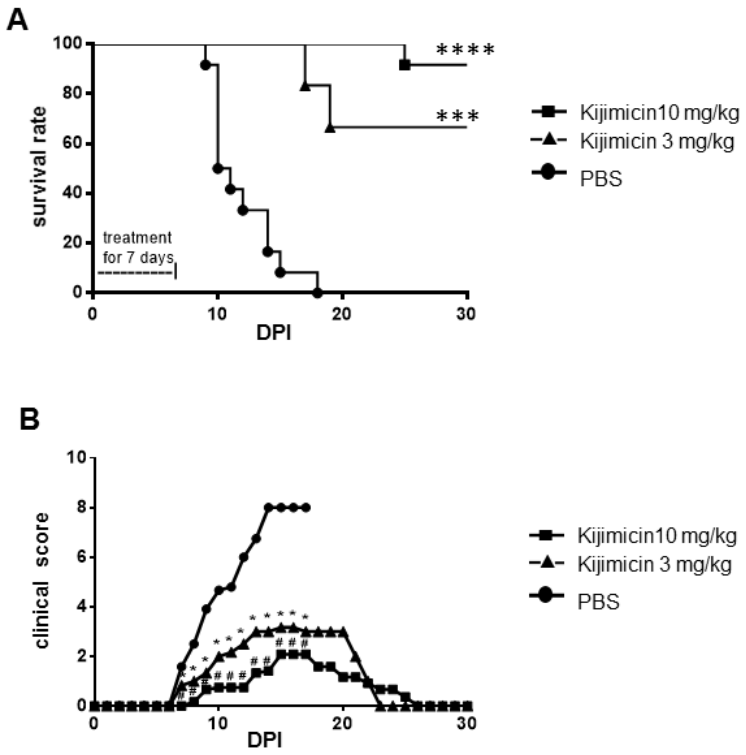

Fig 11. Survival and clinical score of mice infected with *T. gondii* tachyzoites under treatment with kijimycin. Mice infected with 5×10^3 tachyzoites of the *T. gondii* PLK-GFP strain were treated with kijimicin 10 mg/kg/day ($n = 6 + 6$), kijimicin 3 mg/kg/day ($n = 6$), PBS ($n = 6 + 6$) by intraperitoneal injection for 7 consecutive days, clinical scores and survival were observed for 30 days afer nfection. (A) Survival curves were generated by the Kaplan–Meier method. According to the log-rank test, the differences between the PBS and kijimicin were significant (**** Difference between PBS and kijimicin 10 mg/kg/day, $P < 0.0001$; *** Difference between PBS and kijimicin 3 mg/kg/day, $P < 0.0002$). (B) Clinical scores, the clinical scores were analyzed by two-way ANOVA plus Tukey–Kramer post-hoc analysis at the time points indicated (# Difference between PBS and kijimicin 10 mg/kg/day, $P < 0.05$; *Difference between PBS and kijimicin 3 mg/kg/day, $P < 0.05$).

Chapter 3

Studies on the efficacy of metacytofilin against *Toxoplasma* infection

3-1. Introduction

The one important causative agent that causes zoonosis and impacts worldwide is *Toxoplasma gondii*. *T. gondii* is the obligate intracellular protozoan belonged to the phylum apicomplexa. The infection with *T. gondii* is found in almost of the warm-blooded animals and human [Tenter *et al*., 2000; Montoya and Liesenfeld, 2004]. The most of infection in livestock such a poultry, pig and cattle are not a greatest problems, however it can cause reproductive loss in sheep cause of abortion and neonatal mortality or the infected lambs that still alive after birth in this case can be source of infection in human [Dubey and Jones, 2008]. In human, most of infection in healthy adult individual show asymptomatic although the infection causes the severe symptoms in immunocompromised individual hosts particularly in human immunodeficiency virus (HIV) or congenital infection which the treatment are required [Montoya and Liesenfeld, 2004]. The treatments of toxoplasmosis have been not changed for several years, the combination of pyrimethamine and sulfadiazine still use for the first line of treatment [Opremcak *et al*., 1992; Antczak *et al*., 2016]. The treatment procedure particularly in *Toxoplasma* encephalitis, congenital infection, and ocular infection is required for long

duration from weeks to more than a year [Elsheikha, 2008; Harrell and Carvounis, 2014]. The side effects from the drugs might cause discontinue of treatment, and the combination of drug treatment are effective against tachyzoite stage. Because the side effects have been found such as allergic reaction and bone marrow suppression, the treatment usually plus the folic acid to decrease the bone marrow suppression [Opremcak *et al*., 1992; Bosch-Driessen *et al*., 2002]. In case of patients who cannot tolerate to sulfadiazine drugs, the drugs may be replaced with atovaquone, azithromycin, spiramycin or other macrolide groups to avoid the side effects [Andrews *et al*., 2014].

The natural compounds identified from the living organelles are one of the important sources for drug discovery for treatment of toxoplasmosis. Microorganism such as bacteria and fungi can produce the secondary metabolites or toxin that can affect the other living organism. The genus *Metarhizium* (Hypocreales: Clavicipitaceae) is known as insect-pathogenic fungus. It is found in soils and plant rhizome [Driver *et al*., 2000; Keyser *et al*., 2015].

Metacytofilin (MCF) is identified from the fungi *Metarhizium* sp. TA2759 [Iijima *et al*., 1992]. MCF has immunosuppressive effect by significantly inhibiting the mixed lymphocyte culture reaction *in vitro*. Additionally, MCF suppresses the delayed-type hypersensitivity and antibody production in mice. MCF has no effects on growth of the bacterial [Iijima *et al*., 1992], there is no information about anti-parasite activity of MCF. Thus, the aim of this study is to evaluate the anti-*Toxoplasma* activity of MCF *in vitro* and *in vivo*.

3-2. Materials and methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 28-113, 29-45). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering.

Animals

The female mice BALB/c and C57BL/6 mice (6–8 weeks old) were used for evaluating the potency of the drugs. The mice were obtained from Clea Japan, Inc., and were kept in the animal facility at the National Research Center for Protozoan Diseases (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan) under standard laboratory conditions with commercial food and water available *ad libitum*.

Host cell and parasites cultures

Human foreskin fibroblast (HFF) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus 100 U/ml penicillin and 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. African green monkey kidney (Vero) cells were grown in minimum essential medium eagle (MEM, Sigma) plused with 8% FBS and 100 U/ml penicillin, 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

T. gondii, RH-GFP (a green fluorescent protein expressing-RH strain) [Nishikawa *et al*., 2003] and PLK-GFP (PLK strain) [Nishikawa *et al*., 2008] were used in this study. For purification of parasites, the infected cells were washed with cold phosphate-buffered saline (PBS), scraped, and suspended in media. Then, the cells were syringed through a 27-gauge needle to release the parasites. The parasites were filtrated through a 5.0 μm pore filter (Millipore, Bedford, MA), washed twice with 10 ml of media, and centrifuged at 1,000 × *g* for 10 min. After filtration of the parasites, the numbers were counted by a hemacytometer for each experiment.

Chemicals

MCF was kindly provided from Institute of Microbial Chemistry (BIKAKEN). The MCF were prepared at 10 mM in dimethyl sulfoxide (DMSO) and kept at -30°C until use. Sulfadiazine was obtained from Sigma-Aldrich.

Cytotoxic assays

HFF cell suspension (10^5 cells/ml in DMEM plus 10% FBS) was seeded at 100 μl/well in 96-well plates. At 80 to 90% confluence of HFF cells, MCF ranging from 1 to 1,000 μM was added for 72 h. Cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Inc. Japan) was added to the cultures and incubated for 1 to 4 h at 37°C in a 5% CO_2 atmosphere. The absorbance at 450 nm of each well was measured using an MTP-120 micro plate reader (Corona Electric, Ibaraki, Japan). The inhibition of HFF cell proliferation (%) was calculated as the follow [(the average absorbance of cells cultured

with medium alone - the average absorbance of cells treated with MCF) / the absorbance of cells cultured with medium alone] × 100]

Effects of MCF on inhibition of intracellular T. gondii growth

HFF cells, plated at 100 µl/well in 96-well plates (cell suspensions 1×10^5 cells/ml in DMEM supplemented with 10% FBS), were incubated at 37°C in a 5% CO_2 atmosphere. To examine the effects of MCF treatment on the intracellular parasites, RH-GFP and PLK-GFP (5×10^4 tachyzoites per well) were added to the wells for 4 h and the extracellular parasites were washed away. Then, the MCF at final concentrations from 1 to 4 µM (100 µl/well of media) were added for 72 h. The fluorescence intensity of RH-GFP and PLK-GFP were measured using a microplate reader (SH-900, Corona Electric Co., Ltd, Ibaraki, Japan). The growth inhibition of RH-GFP and PLK-GFP (%) was expressed as follows: [(average fluorescence intensity of GFP with medium alone) − (the fluorescence intensity of GFP treated with either of MCF) / (average fluorescence intensity of GFP with medium alone)] × 100. The half maximal inhibitory concentration (IC_{50}) values of MCF or drugs on *T. gondii* were calculated based on three independent experiments performed together by GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA).

Effects of MCF on extracellular T. gondii in vitro

RH-GFP tachyzoites (2×10^5) were pretreated with MCF from 0 to 3 µM, or MEM alone (1 ml/tube) for 1 h at 37°C. Then, the pretreated parasites were added to vero cells on coverslip at 1 ml/well in a 12-well plate (parasites per host cell ratio = 2:1).

At 2-3 h post-infection, the extracellular parasites were washed away and MEM supplemented with 8% FBS was added. After 24 h, the infected cells were washed with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++) twice, and then the infected cells were fixed with 3% paraformaldehyde in PBS++ for overnight at 4°C. Nuclear DNA was labelled with Hoechst 33342 (1:10,000 dilution, Thermo Fisher Scientific Inc., MA, USA) for 15-30 min. The coverslips were washed again two times with PBS++. The coverslips were mounted on a glass slide coated with Mowiol (Calbiochem, San Diego, CA, USA), and the slides were examined using an All-in-one Fluorescence Microscope (BZ-9000, Keyence, Tokyo, Japan). The infection rates were identified base on the green fluorescene which expressed from *T. gondii* RH-GFP per randomly 100 vero cells. The percentage inhibition of the tachyzoites was expressed as follows: [(average infection rate after treatment with medium alone) − (infection rate after treatment with either of MCF) / (average infection rate after treatment with the medium alone)] × 100.

Electron Microscopy

HFF cells were plated in 12 well-plate (1×10^5 cells/1 ml/well). After 24 h, HFF cells were infected with *T. gondii* RH-GFP (2×10^5 cells/well). After 4 h, the cells were washed with PBS once, fresh media contained MCF 1.5 µM and only media were added. After 24 h of treatment, the infected cells were washed with PBS twice, fixed in 3% Glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature. Then, the fixing solution was aspirated and washed with 0.1 M phosphate buffer 3 times. The infected cells were scraped and suspended with 0.1 M phosphate buffer 500 µl, followed by post fixation in 1% osmium tetroxide. Cells were dehydrated in an ethanol series, infiltrated

with LR white resin and polymerized overnight at 60°C. Thin sections (90 nm) were placed on copper grids, stained with 4% uranyl acetate and lead) and observed with HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

Effects of MCF on T. gondii infections in vivo

The female BALB/c mice were inoculated with PLK-GFP 5×10^3 tachyzoites/mouse by intraperitoneal injection. At 24 h post-infection, the mice were intraperitoneally injected with MCF 30 mg/kg/day (n = 6), 10 mg/kg/day (n = 12), 3 mg/kg/day (n = 6) or PBS mg/kg/day (n = 12) via the intraperitoneal route or 400 mgl sulfadiazine via the drinking water as a standard treatment for 7 days. To further evaluate the anti-*Toxoplasma* activity of the MCF, the mice intraperitoneally infected with 5×10^3 PLK-GFP strain, the infected-mice were treated with MCF 30 mg/kg/day (n = 6), 10 mg/kg/day (n = 6) and PBS (n = 6) per oral route. The clinical signs, morbidity, and mortality including the body weight of each mouse were daily observed until 30 days of post infection (dpi) in both trial. The clinical signs were scored from 0 to 10 scores based on the observing of hunching, piloerection, worm-seeking behavior, ptosis, sunken eyes, ataxia, latency of movement, deficient evacuation and touch reflexes, and lying on belly [Hermes *et al*., 2008; Carroll *et al*., 2010].

Effects of MCF on pregnant mice toxoplasma infection

Virgin female mice were housed with males at 6–8 weeks of age, and when a visible vaginal plug was noted I designated this as day 0 of pregnancy (E0). Female mice were inoculated intraperitoneally with strain PLK *T. gondii* tachyzoites (5×10^3/mouse) at

day E3. At 24 h after the infection, the infected mice were treated with MCF at 10 mg/kg (n = 7) or PBS (n = 6) per oral route for 7 days. Each female was separated alone before giving birth to offspring. The survival and body weight of offspring were monitored for 30 days after birth. The clinical signs, morbidity and mortality of dams were also observed until 30 dpi.

Statistical analysis

All data were analyzed using GraphPad Prism 5 software (GraphPad Software Inc.) Data were shown as representative of mean ± SD. Statistical analyses, the group comparisons were performed using a one-way or two-way analysis of variance (ANOVA) followed by the Tukey–Kramer test or Bonferroni's multiple comparisons test for group comparisons. The Kaplan–Meier method, and statistical comparisons between the curves using the log-rank method were used for survival curves. A P value of < 0.05 was considered statistically significant are shown as asterisks or symbol and defined in each figure legend together with the name of the statistical test.

3-3. Results

Effects of MCF on inhibition T. gondii growth and HFF cell proliferation

At first, the inhibition of MCF on the growth of intracellular and extracellular *T. gondii*, type I strain RH-GFP, was evaluated (Fig. 12). The IC_{50} of MCF on intracellular and extracellular RH-GFP parasites was 1.2 μM and 2.4 μM, respectively. Since the IC_{50} of MCF on HFF cells was 167.8 μM, the selectivity index (SI) on intracellular and

extracellular parasites was 141 and 69.3, respectively. These results showed that the effects of MCF against intracellular type I strain of *T. gondii* were slightly higher than those against extracellular parasites. Furthermore, the inhibition of MCF on intracellular *T. gondii*, type II strain PLK-GFP, was also evaluated because this strain was used in an *in vivo* study. The IC_{50} and SI of MCF on intracellular PLK-GFP parasites were 214.5 nM and 782, respectively. This result indicated that MCF has higher anti-type II strain of *Toxoplasma* activity compared with the type I strain.

Base on the observation with electron microscopy, the changing of ultrastructure were noticed under the treatment with MCF. In the MCF-treated tachyzoites, the electron-dense coat structure, the generation of vacuole-like structures and cellular debris which were filled inside the parasite cells were found. This observation might be the beginning of parasite disruption (Fig. 13).

Effects of MCF on T. gondii infection in vivo

As the first trial, the infected mice were intraperitoneally treated with MCF, and the mouse survival and clinical symptoms were observed for 30 dpi (Fig. 14). As a standard treatment, the infected mice were treated with 400 mg/l sulfadiazine via the drinking water. In the infected-mice that were treated with sulfadiazine and MCF at 30 mg/kg/day, all mice survived while the all PBS-injected mice died within 18 dpi (Fig. 14A). In the treatment with MCF at 10 mg/kg and 3 mg/kg, the mouse survival rates were 83.9% and 33.33%, respectively (Fig. 14A). The clinical signs were observed at 7 dpi and its score increased until 17 dpi in PBS-injected mice (Fig. 14B). The mice treated with MCF at 3 mg/kg/day showed the clinical signs from 7 dpi, and the mice with the

symptom did not recover until the end of experiments. Although the mice which were treated with MCF at 10 mg/kg/day showed clinical sign from 9 dpi, they recovered from 18 dpi. Treatment of the infected-mice with MCF at 30 mg/kg/day significantly suppressed the symptoms throughout the experiment as sulfadiazine treatment did. As shown Fig. 14C, MCF treatment via intraperitoneal route decreased the parasite number in the brain in a dose-dependent manner.

To validate the rout of administration, the infected mice were treated with PBS and MCF at 30 and 10 mg/kg/day per oral route (Fig. 15). The survival rate of mice treated with MCF at 30 and 10 mg/kg/day was 100% while all PBS-administrated mice died within 11 dpi (Fig. 15A). The clinical signs of the MCF-treated mice were significantly lower than those of PBS-administrated animals (Fig. 15B). Remarkably, no clinical sign was seen in the mice treated with MCF at 30 mg/kg/day. Although the parasite DNA was detected in the brain of mice treated with MCF at 10 mg/kg/day, treatment with MCF at 30 mg/kg/day significantly inhibited the parasite burden in the brain (Fig. 15C) as similar levels of sulfadiazine treatment (Fig. 14C). These results showed that treatment with MCF via oral route efficaciously controlled *T. gondii* infection in mice.

Next, effects of MCF in pregnant mice were evaluated (Fig. 16). All mice survived in MCF-treated group at 10 mg/kg/day per oral route while one mouse died at 10 dpi in PBS-treated group (Fig. 16A). Clinical signs in PBS-treated mice were observed from 8 dpi until the time point of experiment. Although, in MCF-treated group, one mouse showed clinical signs starting from 18 dpi, the clinical signs of MCF-treated group were significantly lower than those of PBS-treated group from 8 dpi (Fig. 16B). In the mice treated with MCF, only one mouse failed maintenance of pregnancy, resulting in

85.7% birthrate (Fig. 16C). On the other hand, all mice did not have the offspring in PBS-treated group. Thus, this result indicated anti-*Toxoplasma* activity of MCF in the pregnant mice.

3-4. Discussion

The combination of pyrimethamine and sulfadiazine is the mainstay of treatment for toxoplasmosis. However, this treatment is effective on the tachyzoite stage, and shows several side effects, resulting in discontinuous treatment. Currently, alternative drugs such as clindamycin, clarithromycin, atovaquone, azithromycin, spiramycin and clindamycin are available if sulfonamide cannot be used. Macrolide such as spiramycin has been used for pregnant women with acute toxoplasmosis to reduce transmission of the parasites to the fetus [Montaya, 2004]. Because spiramycin cannot cross through the placenta but concentrated in it, this drug can prevent the vertical transmission of *T. gondii*. In Europe and the USA, treatment of pregnant women with spiramycin immediately after positive diagnosis is recommended, resulting that approximately 60% reduction of the vertical transmission [Montaya, 2004]. Treatment with pyrimethamine is contraindicated during the first trimester of pregnancy because it is associated with teratogenic [Rorman *et al*., 2006]. Azithromycin, macrolide antibiotic, can reduce the severity of congenital infection [Tamaru *et al*., 2011]. Treatment with azithromycin alone or combination with pyrimethamine have been used for treatment of *Toxoplasma* encephalitis in patients with AIDS [Chang, 1996; Wiselka *et al*., 1996] and for toxoplasmic retinochoroiditis [Balaskas *et al*., 2012]. Clindamycin, lincosamide

antibiotic, which disturbs parasite protein synthesis, is used for controlling *Toxoplasma* retinochoroiditis [Harrell and Carvounis, 2014]. However, treatment with clindamycin combined with pyrimethamine for acute toxoplasmosis is not effective, and causes some side effects [Katlama *et al*., 1996]. Because current drugs for treatment of toxoplasmosis have toxicity, limited efficacy and can control only tachyzoite stage [Alday and Doggett, 2017]. Thus, searching for new potential drugs with more effective and lower side effects are challenging and urgently needed [Antczak *et al*., 2016]. The natural sources from the plants, microorganisms such a bacteria and fungi have a potential having anti-*Toxoplasma* activity [Galm and Shen, 2007]. In my preliminary study in an *in vitro* using a chemical library generated from fungi, I found a potential of MCF on anti-*Toxoplasma* activity. Therefore, I further evaluated the effects of MCF against *T. gondii* infection in this study.

MCF inhibited intracellular growth of type I and type II *T. gondii* (IC_{50} of RH-GFP, 1.2 µM; IC_{50} of PLK-GFP, 214.5 nM). My previous study found that IC_{50} of azithromycin and spiramycin against the growth of type I *T. gondii* was 28.2 µM and 85.1 µM, respectively. When compared the SI values which related to the safe of drug treatment calculated by ratio of inhibition host cell proliferation IC_{50} to inhibition parasite growth IC_{50}, the efficacy of MCF was higher than that of azithromycin and spiramycin (SI of MCF, 141; SI of azithromycin, 2.6, SI of spiramycin, 0.7). MCF also affected extracellular *T. gondii* by inhibiting the parasite invasion into host cells. This result suggested that MCF might affect the cell membrane of the parasites or the parasite molecule involved in its invasion. Furthermore, the ultrastructure of *T. gondii* was changed by the MCF treatment. The analysis with transmission electron microscope

showed that MCF induced the generation of the electron-dense coat structure, vacuole-like structure and cellular debris inside the parasite cells, suggesting the beginning of the parasite disruption and lysis.

In vivo experiments, the treatment of non-pregnant mice with MCF via intraperitoneal route at 30 mg/kg/day improved the clinical signs and survival rate following *T. gondii* infection. Remarkably, oral treatment of non-pregnant mice with MCF also effectively controlled *T. gondii* infection as sulfadiazine treatment did. Because I detected MCF in blood of the orally treated and intraperitonelly treated mice (data not shown), MCF has an ideal pharmacodynamics. Importantly, oral administration with MCF was also effective against *T. gondii* infection in pregnant mice. Although the non-treated mice failed to have offspring, MCF-treated animal could maintain pregnancy and give birth. Therefore, MCF inhibited the parasite transmission from the mother to fetus. Previously, the toxicity of MCF was tested, and 100 μg/ml MCF showed no cytotoxic effects to mouse lymphocytic leukemia cells (L1210), mouse tumor cell lines (EL-4) and IMC carcinoma cells [Iijima *et al*., 1992]. Furthermore, intraperitoneal injection of ICR mice with MCF at 200 mg/kg did not cause any toxicity to ICR mice [Iijima *et al*., 1992]. According to the previous results and the present study, treatment with MCF may be safe under the experimental condition in this study.

3-5. Summary

Because MCF has been shown as immunomodulator, its pharmacological property

may regulate the inflammation during acute phase of *T. gondii* infection. Additionally, the direct effects of MCF to *T. gondii* can be considered to the anti-*Toxoplasma* activity. The present findings indicate that MCF will be one candidate of the new compound for treatment of toxoplasmosis. Since I did not elucidate the mode of action of MCF, further study will be required for providing useful information to combat *T. gondii* infection.

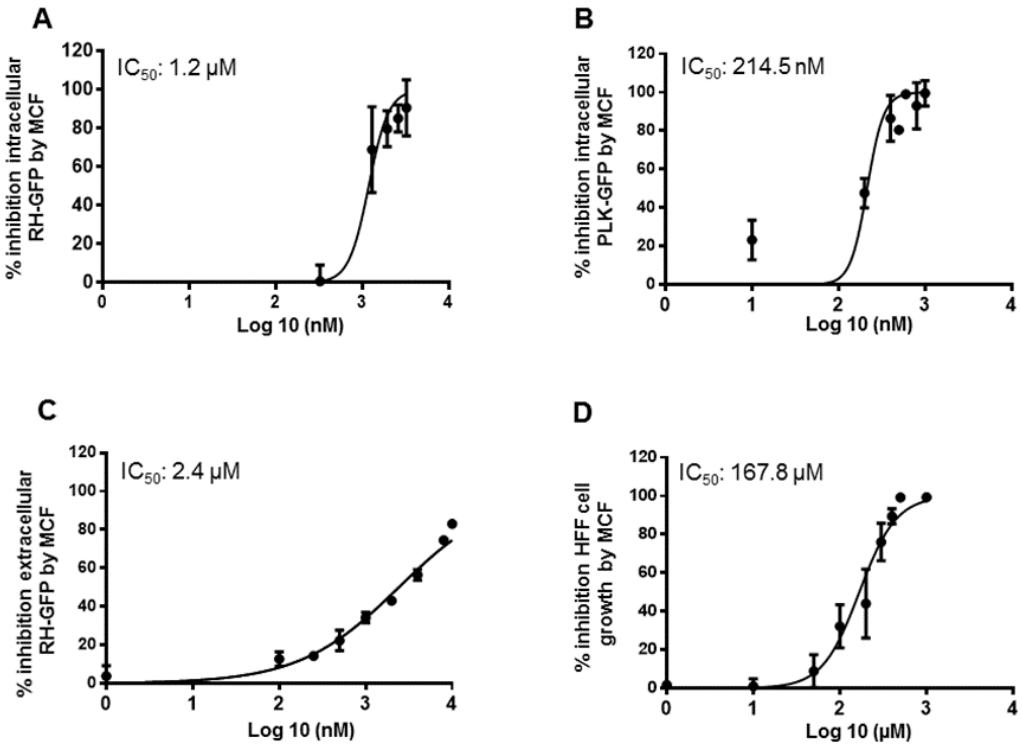

Fig 12. Anti-*Toxoplasma* activity and inhibition of HFF cell growth. Anti-*Toxoplasma* activity of MCF on intracellular parasites RH-GFP (A) and PLK-GFP (B). The RH-GFP and PLK-GFP-infected HFF cells were treated with MCF for 72 h at different concentrations from 1 µM to 4 µM. Data represent the mean values ± SD for three independent experiments. The IC_{50} values of MCF on RH-GFP and PLK-GFP were 1.2 µM and 214.5 nM, respectively. (C) Anti-*Toxoplasma* activity of MCF on extracellular parasites RH-GFP. The parasites were pretreated with MCF at different concentrations from 0 µM to 3 µM for 1h before allowed the parasites infected the vero cell. The IC_{50} of MCF on inhibition of *T. gondii* infection into vero cells was 2.4 µM. (D) Effects of MCF on growth of HFF cells. HFF cells were exposed to MCF at different concentrations from 1 to 1,000 µM. The IC_{50} values of MCF on HFF cells was 167.8 µM.

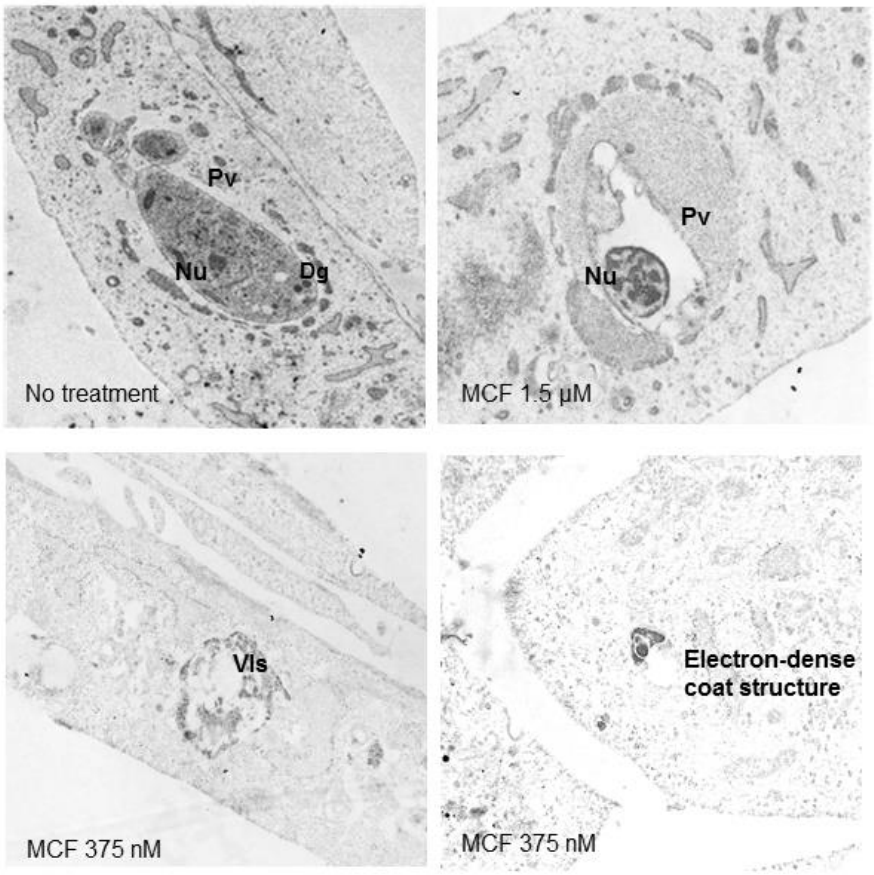

Fig 13. Tranmission electron microscographs of intracellular parasites. The infected vero cells were treated with media (no treatment) or MCF (375 nM and 1.5 µM) for 24 h, then the infected cells were analysed by tranmission electron microscope. Pv, parasitophorous vacuole; Nu,nucleus; Dg, dense granule; Vls, vacuole-like structure.

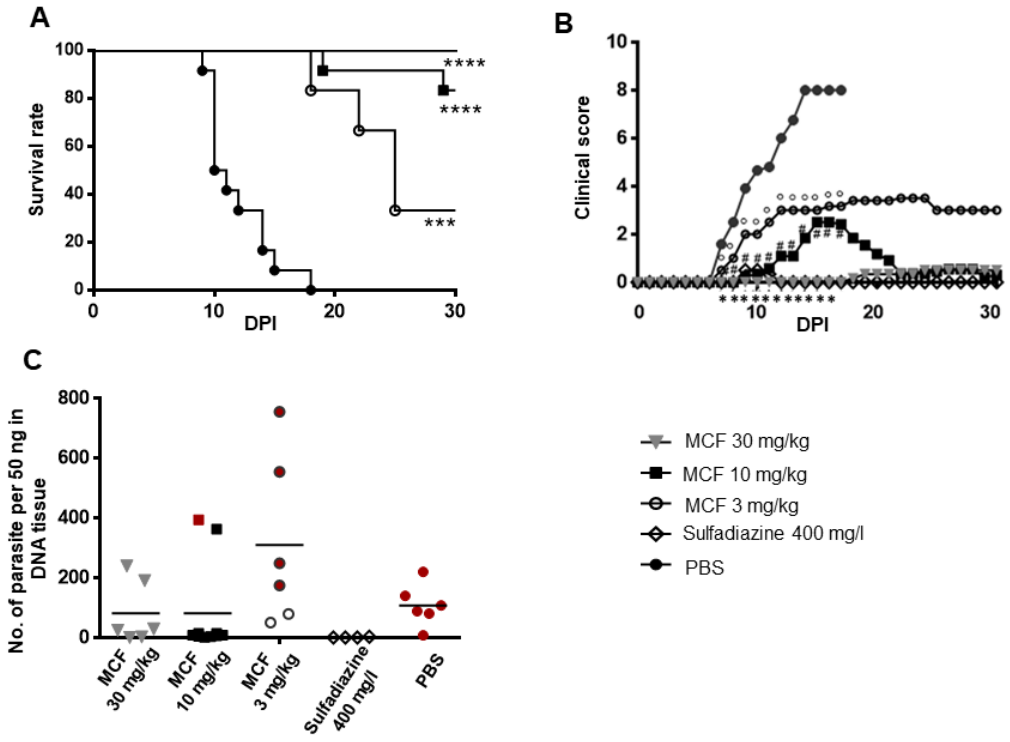

Fig 14. Survival, clinical scores and parasite burden of *T. gondii*-infected mice treated with MCF by intraperitoneal route. Mice were infected with PLK-GFP (5×10^3 tachyzoites), after 24 h the infected-mice were treated with MCF 30 and 3 mg/kg/day ($n = 6$), MCF 10 mg/kg/day ($n = 6 + 6$), PBS ($n = 6 + 6$) by intraperitoneal route (IP) and sulfadiazine 400 mg/l via drinking water ($n = 6$) as the control group for 7 days and observed morbidity and motility for 30 day-post infection (dpi). (A) Survival rate were generated by the Kaplan–Meier method, regarding to the log-rank test, the differences among the PBS, MCF treatment and sulfadiazine were significant (****Difference between PBS and MCF treatment at 30, or 10 mg/kg/day or sulfadiazine treatment, $P < 0.0001$; ***difference between PBS and MCF treatment 3 mg/kg/day, $P < 0.001$). (B) Clinical scores were given from 0 (no signs) to 10 (all signs). The clinical scores were

analyzed by two-way ANOVA plus Tukey–Kramer post-hoc analysis at the time points indicated (*Difference among PBS, MCF treatment 30 mg/kg/day and sulfadiazine 400 mg/l, $P < 0.05$; # Difference between PBS and MCF treatment at 10 mg/kg/day, $P < 0.05$; °Difference between PBS and MCF treatment at 3 mg/kg/day, $P < 0.05$). (C) Parasite burden, the number of parasites in 50 ng of tissue DNA per individual (symbols) and the mean levels (horizontal lines) are indicated, the red symbols were the parasite burden from the death mice. No significant difference of parasite burden from brain tissue of survival mice between MCF, sulfadiazine and PBS-treated group was observed by one way ANOVA plus Tukey–Kramer post-hoc analysis.

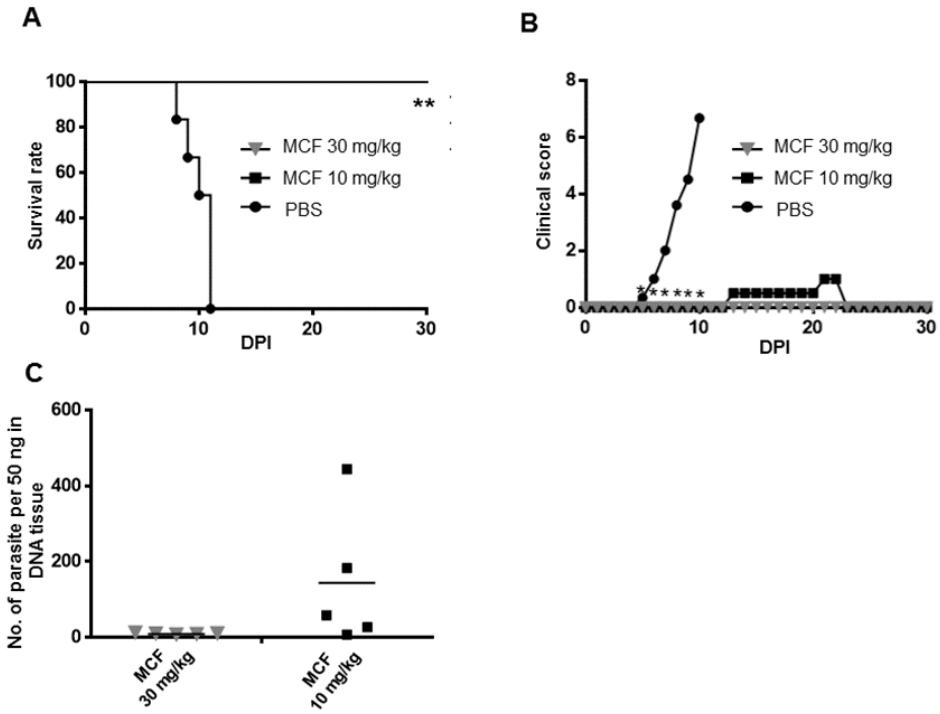

Fig 15. Survival, clinical scores and parasite burden of *T. gondii*-infected mice treated with MCF per oral route. Mice were infected with *T. gondii* PLK-GFP 5×10^3 tachyzoites ($n = 6$) after infected 24 h the infected mice were treated with the drugs MCF 30 and 10 mg/kg/day, PBS as the control group per oral route (PO) for 7 consecutive days. (A) Survival rate were generated by the Kaplan–Meier method, regarding to the log-rank test, the differences between the PBS and MCF treatment were significant (**Difference among PBS and MCF treatment at 30 or 10 mg/kg, $P < 0.001$). (B) Clinical scores, the data represent the mean of each treatment group. The scores were given from 0 to 10 score, the clinical scores were analyzed by two-way ANOVA plus Tukey–Kramer post-hoc analysis at the time points indicated (*Difference among PBS, MCF treatment at 30 and 10 mg/kg/day, $P < 0.05$). (C) Parasite burden, the number of parasites in 50 ng of tissue DNA per individual (symbols) and the mean levels (horizontal lines) are indicated. No significant difference between the two groups was observed by a student's *t*-test.

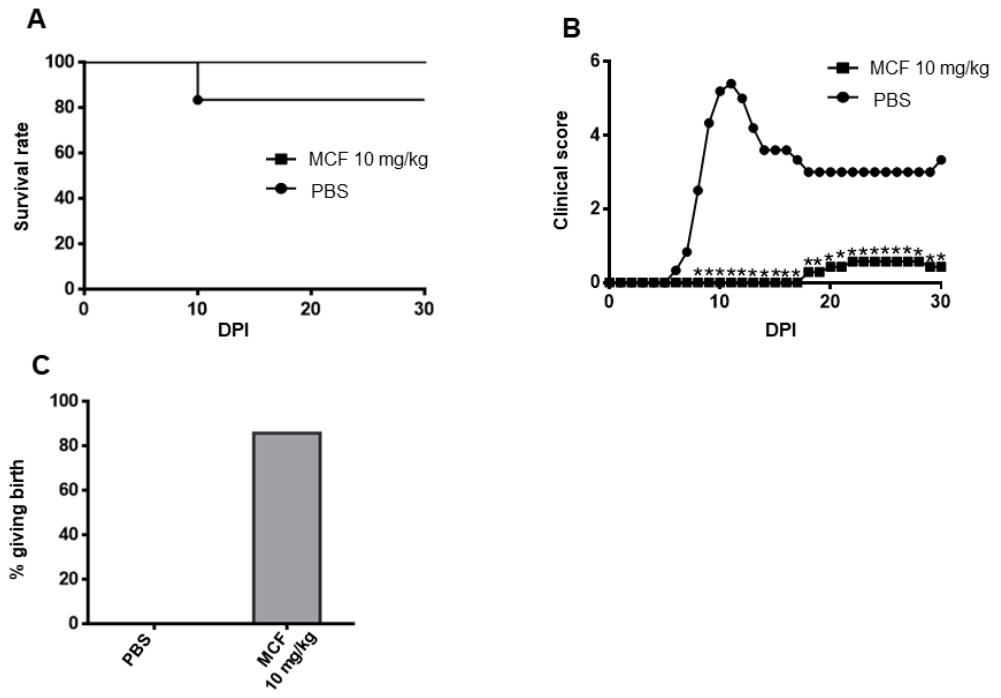

Fig 16. Survival, clinical scores and percent of birthrate of *T. gondii*-infected pregnant mice treated with MCF per oral route. Pregnant mice were infected with *T. gondii* PLK (5×10^3 tachyzoites) at day 4 of gestation. After 24 h, the infected-mice were treated with MCF at 10 mg/kg/day (n = 7) and PBS (n = 6) as the control group per oral route for 7 consecutive days. (A) Survival rate were generated by the Kaplan–Meier method, regarding to the log-rank test. There was no significant differences between the PBS and MCF treatment. (B) Clinical scores, the data represent the mean of each treatment group. The scores were given from 0 to 10 score, the clinical scores were analyzed by two-way ANOVA plus Bonferroni's multiple comparisons test at the time points indicated (*Difference between PBS and MCF treatment at 10 mg/kg/day, $P < 0.05$). (C) Percent of birthrate of the infected dams. The birthrate of MCF-treated group was 85.7% while all mice failed to make offspring in PBS-treated group.

General discussion

Toxoplasmosis is one of the most important disease in public health. Because *T. gondii* mainly infects by ingestion the contaminated tissue cysts or sporulated oocysts, the good personal hygiene is required for preventing the transmission of *T. gondii* such as avoiding the consumption raw or undercooked meat, washing fruit and vegetables, washing hand or using gloves when handling the cat [Lopez *et al*., 2000]. The chemicals for toxoplasmosis treatments should be provided in case of immunocompromised host which cause severe life-threatening, congenital toxoplasmosis [Montoya and Liesenfeld, 2004]. The ideal chemicals for treatment should have potential against both acute and chronic stages of the infection, including effects on tissue cysts, low toxic compared to the standard treatments [Alday and Doggett, 2017]. However, the drugs currently used do not fill up these properties. Find out the novel compounds which have anti-*Toxoplasma* activity are encouraged instead of the old regimens having side effects and limitation of efficacy [Antczak *et al*. 2016]

Plants and microorganism such a bacteria and fungi can produce the secondary metabolites that possess bioactivity with several pharmacological properties; antibacterial, antifungi, antiparasite, prevention competing other species. Besides, the secondary metabolites are also focused on use for human medicine [Oksman-Caldentey and Inze, 2004]. Until now, several crude extracts from natural products, plants, marine organism, and toxin have been tested for anti-*Toxoplasma* activity. [Sepulveda-Arias *et al*., 2014]. The main purpose of this study was to evaluate the crude extracts from three

piperaceae plants; *P. betle*, *P. nigrum*, and *P. sarmentosum* and microorganism-derived compounds; kijimicin and MCF on anti-*Toxoplasma* activity in an *in vitro* and *in vivo.* The results have been successfully provided that plants and microorganism sources contained the anti-*Toxoplasma* activity.

In chapter 1, the crude ethanol extracts from three piperaceae plants; *P. betle*, *P. nigrum*, and *P. sarmentusum* were evaluated on their anti-*Toxoplasma* activity. The results showed that piperaceae plants particular *P. betle* crude extract had anti-*Toxoplasma* activity by inhibiting intracellular and extracellular parasites. According to the IC_{50}, the efficacy of *P. betle* crude extract on intracellular parasites was higher than that of sulfadiazine in both type I and II strains of *T. gondii* (IC_{50} of *P. betle* crude extract on RH-GFP: 23.2 µg/ml and PLK-GFP: 21.4 µg/ml, IC_{50} of sulfadiazine on RH-GFP: 99.4 µg/ml and PLK-GFP: 22.3 µg/ml). All crude extracts at concentration 25 µg/ml were more effective on extracellular parasites than sulfadiazine at 1 mg/ml. Especially, *P. betle* crude extract eradicated the extracellular parasites to infect the host cells because of the destruction of parasite cell membrane. *In vivo*, the infected mice (1×10^3 and 1×10^5 PLK tachyzoites) were treated with *P. betle* crude extract. Particularly, treatment with *P. betle* crude extract at 400 mg/kg significantly increased mouse survival rate (100%) compared with PBS-treated group (survival rate: 0%). Therefore, *P. betle* crude extract can ameliorate toxoplasmosis in mice during the acute infection. The mechanism of *P. betle* crude extract on *T. gondii* is still under investigation. *P. betle* leaves contain several phytochemicals; alkaloids, terpenes, anthraquinones, flavonoids, tannins, saponins and steroids [Al-Adhroey *et* al., 2010]. The phytochemical compounds such as alkaloids and

flavonoids have antiplasmodial activities [Al-Adhroey *et al*., 2010]. Therefore, alkaloids and flavonoids that found in *P. betle* crude extract might be active against *T. gondii*.

In chapter 2, kijimicin obtained from bacterium *Actinomadura* sp. MI215-NF3 [Takahashi *et al*., 1990] was tested on anti-*Toxoplasma* activity. The results provided that kijimicin inhibited *T. gondii* growth. The efficacy on extracellular *T. gondii* was higher than that on intracellular parasites (IC_{50} on intracellular *T. gondii*: 45.6 nM and extracellular parasite: 216.6 pM). Kijimicin and monensin were more effective than the reference drugs for treatment of toxoplasmosis (IC_{50} of monensin: 1.3 nM, IC_{50} of azithromycin: 28.2 µM, IC_{50} of clindamycin: 238.5 nM, IC_{50} of spiramycin: 85.1 µM, and IC_{50} of sulfadiaizne: 397.2 µM). Additionally, kijimicin could control *T. gondii* infection in mice because the survival rate and clinical signs of the mice were improved. The infected mice that were treated with kijimicin at 10 and 3 mg/kg/day showed 91.7% and 66.7% survival, respectively, while all mice died within 18 dpi in the PBS-treated group. Kijimicin might have the same mode of action as monensin because the changing in morphology such as cell swollen, vacuole-like structures, loss of normal function and lysis in final were found in kijimicin and monensin treatment [Lavine and Arrizabalaga, 2012]. Moreover, kijimicin may induce oxidative stress in mitochondria. As shown in monensin effects, the production of reactive oxygen species will disturb membrane potential and disrupt mitochondrial morphology leading to the death of parasites [Charvat and Arrizabalaga, 2016]. Although kijimicin and monensin have high efficacy to inhibit parasite growth, the treatment have to be considered because of the toxicity to the host.

In chapter 3, the potency of MCF on anti-*Toxoplasma* activity was evaluated. MCF is identified from the fungi *Metarhizium* sp. TA2759, and has immunosuppressive

effect [Iijima *et al*., 1992]. In this study, MCF inhibited intracellular *T. gondii* of type I and II strains (IC_{50} on RH-GFP: 1.2 µM and PLK-GFP: 214.5 nM) and affected to extracellular *T. gondii* by inhibiting the parasite invasion into host cells. Thus, MCF might affect the cell membrane of the parasites or the parasite molecule involved in its invasion. MCF showed lower cytotoxicity to host cells (IC_{50}: 167.8 µM), and the SI value of MCF was higher than azithromycin and spiramycin (SI of MCF, 141; SI of azithromycin, 2.6, SI of spiramycin, 0.7). MCF controlled *T. gondii* infection in mice via both intraperitoneal and per oral routes. Remarkably, MCF protected pregnant mice against *T. gondii* infection. Treatment of the infected-pregnant mice with MCF at 10 mg/kg provided normal birth while the PBS-treated animals did not birth. Because MCF are immunomodulator, its pharmacological property may regulate the inflammation during acute phase of *T. gondii* infection. In addition, the direct effects of MCF to *T. gondii* can be considered for the anti-*Toxoplasma* activity while further study on the mode of action by MCF treatment is needed. MCF induced the generation of vacuole-like structure and cellular debris which were filled inside the parasite cells. This observation may be the beginning of the cell disruption and lysis of the parasites. The present findings indicate that MCF will be one candidate of the new compound for treatment of toxoplasmosis.

From the present results, *P. betle* crude extract, kijimicin and MCF had anti-*Toxoplasma* activity in both *in vitro* and *in vivo*, therefore *P. betle* crude extract, kijimicin and MCF will be the candidates for development of anti-*Toxoplasma* drug.

General summary

Since current anti-*Toxoplasma* drugs have limited efficacy and side effects, searching on the new substances should be required for an alternative of drug treatments. Natural products such as plants and microorganism are the major sources of drug discovery because the secondary metabolites have diversity of bioactive and pharmacological properties. In this study, I proved that natural products obtained from plants, bacteria and fungi are important sources of substances having anti-*Toxoplasma* activity.

In chapter 1, *P. betle* crude extract had anti-*Toxoplasma* activity by inhibiting intracellular *T. gondii* growth. Moreover, crude extracts of *P. betle, P. nigrum* and *P. sarmentusum* inhibited *T. gondii* invasion. *In vivo*, treatment with *P. betle* crude extract improved the clinical signs and symptoms in the infected mice at the acute phase of the infection and increased the survival rate of the infected mice. These results indicate that *P. betle* crude extract contains active ingredients with potential for treating toxoplasmosis.

In chapter 2, microorganism-derived compounds were tested on anti-*Toxoplasma* activity. Kijimicin, which belongs to polyether ionophore group, is produced from bacterium *Actinomadura* sp. MI215-NF3. Kijimicin had anti-*Toxoplasma* activity and was more effective to extracellular than intracellular parasites (IC_{50} on extracellular and intracellular RH-GFP: 216.6 pM and 45.6 nM). Furthermore, the efficacy of kijimicin on intracellular parasites was higher than that of azithromycin, clindamycin, spiramycin and

sulfadiazine. Kijimicin induced formation of vacuole-like structures in *T. gondii,* which was also found in monensin treatment. Importantly, treatment of *T. gondii*-infected mice with kijimicin at 10 and 3 mg/kg/day significantly increased mouse survival (survival rate: 91.7% and 66.7%, respectively) and decreased the clinical signs during the acute phase of the infection. The results showed that kijimicin inhibited intracellular parasite growth and invasion of the extracellular parasites into the host cells, and protected mice against *T. gondii* infection.

In chapter 3, MCF produced from the fungi *Metarhizium* sp. TA2759 was evaluated on anti-*Toxoplasma* activity. MCF inhibited *T. gondii* growth both in an *in vitro* and *in vivo*. *In vitro,* MCF had inhibition effects on intracellular and extracellular parasites (IC_{50}: 1.2 μM and 2.4 μM, respectively). *In vivo,* MCF moderated the parasite infection in mice model. The treatment with MCF by intraperitoneal and per oral administration increased survival rate and improved the clinical signs. Remarkably, MCF inhibited the parasites to pass through the placenta and to infect the fetus. In contrast, all mice failed pregnancy in PBS-treated group. Therefore, MCF will be the one candidate that affects to *T. gondii* and protect the congenital infection during pregnant period.

The present studies conclude that *P. betle* crude extracts, kijimicin and MCF have anti-*Toxoplasma* activity. These materials will be important source for development of novel drug for treatment of toxoplasmosis.

和文要約

トキソプラズマ症は免疫不全あるいは先天性感染で重篤な症状を起こし、公衆衛生上も重要な感染症である。重篤な臨床症状や先天性感染に対しては治療を必要とする。現在の治療法はピリメタミンとサルファ剤の併用であるが、治療効果が低いことと副反応が問題となっている。それ故、新規の治療薬の開発が必要とされている。植物や微生物由来の天然物は多様な生物活性と薬理活性を有するため、薬剤開発の重要な資源となる。本学位論文の目的は、植物資源由来としてタイ産コショウ科植物の抽出物、細菌資源由来としてキジマイシン、真菌資源由来としてメタサイトフィリンに着目し、in vitro と in vivo にて抗トキソプラズマ活性を評価した。

第一章では、タイ産コショウ科植物抽出物の抗トキソプラズマ活性を評価した。薬草療法や天然薬草抽出物は様々な寄生虫感染症に対する対処療法として使用されており、トキソプラズマ症の治療においても既存薬の副反応を軽減できる可能性を持つ。そこで、タイ産コショウ科植物（*Piper betle*、*P. nigrum*、*P. sarmentosum*）のエタノール抽出物に対する抗トキソプラズマ活性を検証した。In vitro における抽出物の抗トキソプラズマ活性を調べたところ、*P. betle* 抽出物の活性が最も高く、ヒト繊維芽細胞（HFF 細胞）内におけるトキソプラズマの増殖を抑制した（RH-GFP 株に対する IC_{50}: 23.2 μg/ml, PLK-GFP 株

に対する IC_{50}: 21.4 μg/ml）。次に、PLK 株（1,000 タキゾイト）を感染させたマウスに対し腹腔内投与により *P. betle* 抽出物で７日間治療したところ、未治療マウスと比較してマウスの生存率の上昇が確認された（100%：400 mg/kg/day 投与、83.3%：100 mg/kg/day 投与、33.3%：25 mg/kg/day 投与、33.3%：未治療）。さらに 400 mg/kg/day 投与で治療したマウスは、100,000 タキゾイト感染に対しても 100%の生存を示した。この結果は、*P. betle* 抽出物がトキソプラズマ症の治療用の薬草として使用できる可能性を示している。

第二章では、*Actinomadura* sp. MI215-NF3 より同定されたキジマイシンを評価した。キジマイシンはイオノフォアに属し、グラム陽性細菌に対する抗菌作用や急性および慢性感染期の免疫不全ウイルスに対する抗ウイルス作用を持つ。さらに、イオノフォアに属するモネンシンやサリノマイシンと比較して、キジマイシンは鶏コクシジウム症の原因原虫 *Eimeria tenella* に対しても有効である。そこで、キジマイシンの抗トキソプラズマ作用をモネンシン、トキソプラズマ治療薬（アジスロマイシン、クリンダマイシン、スピラマイシン、サルファ剤）と比較した。HFF 細胞内トキソプラズマ（RH-GFP 株）に対するキジマイシン、モネンシン、クリンダマイシンの IC_{50} は、それぞれ 45.6 nM、1.3 nM、238.5 nM であった。さらに、キジマイシンとモネンシンは細胞外原虫にも作用し、トキソプラズマの宿主細胞侵入を阻害した（キジマイシンの IC_{50}: 216.6 pM, モネンシンの IC_{50}: 513.1 pM）。アジスロマイシンとスピラマイシンについては、本実験では有効な抗トキソプラズマ作用は認められなかった。電子顕微鏡による形態観察の結果、キジマイシンで処理したトキソプラズマは細胞の

膨化と多数の液胞形成が認められ、モネンシンで処理した場合と同様の結果が得られた。次に、PLK 株（5,000 タキゾイト）を感染させたマウスに対し腹腔内投与によりキジマイシンで７日間治療したところ、未治療マウスと比較してマウスの生存率の上昇が確認された（91.7%：10 mg/kg/day 投与、66.7%：3 mg/kg/day 投与、0%：未治療）。また、キジマイシン投与マウスでは感染 7～17 日で臨床症状の軽減が認められた。この結果により、トキソプラズマ阻害剤としてのキジマイシンの有効性が示された。

第三章では、*Metarhizium* sp. TA2759 より同定されたメタサイトフィリンの抗トキソプラズマ活性を評価した。In vitro 解析の結果、細胞内および細胞外原虫（RH-GFP 株）に対する IC_{50}はそれぞれ 1.2 μM（選択毒性：141）と 2.4 μM（選択毒性：69.5）であった。電子顕微鏡による形態観察の結果、メタサイトフィリンで処理したトキソプラズマは高電子密度の構造物や細胞の残骸の蓄積、液胞様構造の形成が認められた。次に、PLK 株（5,000 タキゾイト）を感染させたマウスに対し腹腔内投与によりメタサイトフィリンで７日間治療したところ、未治療マウスと比較してマウスの生存率の上昇が確認された（100%：30 mg/kg/day 投与、83.3%：10 mg/kg/day 投与、33.3%：3 mg/kg/day 投与、0%：未治療）。さらに、PLK 株（5,000 タキゾイト）を感染させたマウスに対し経口投与によりメタサイトフィリンで７日間治療したところ、未治療マウスと比較してマウスの生存率の上昇が確認された（100%：30 mg/kg/day 投与、100%：10 mg/kg/day 投与、0%：未治療）。上記の実験系でサルファ剤 400 mg/L（自由飲水）で７日間標準的な治療した場合もマウスの生存率は 100%であったこと、メ

タサイトフィリンで治療したマウスは臨床症状も軽減したことから、メタサイトフィリンの抗トキソプラズマ活性の有効性が示された。そこで、メタサイトフィリンの妊娠期感染に対する有効性を検証した。妊娠３日に PLK 株（5,000 タキゾイト）を感染させたすべてのマウスは正常な妊娠を維持することはできなかった。その一方、妊娠期に感染させたマウスに対し経口投与によりメタサイトフィリンで７日間治療したところ、妊娠期の臨床症状の軽減が認められ、妊娠率が 85.7%となり正常な出産も確認した。以上より、メタサイトフィリンの新規トキソプラズマ症治療薬としての有効性が示された。

本研究の成果により、タイ産コショウ科植物 *P. betle* の抽出物、キジマイシンおよびメタサイトフィリンの抗トキソプラズマ活性が証明された。これらの天然資源や化合物は新規のトキソプラズマ治療薬の開発に有効な研究資源となり、今後の薬剤開発の進展が期待される。

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