Effects of Thai piperaceae plant extracts on *Neospora caninum* infection

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Abstract

Neosporosis has a worldwide distribution and causes economic losses in farming, particularly by increasing the risk of abortion in cattle. This study investigated the effects of Thai piperaceae (*Piper betle, P. nigrum*, and *P. sarmentosum*) extracts on *Neospora caninum* infections *in vitro* and *in vivo*. In an *in vitro* parasite growth assay based on the green fluorescent protein (GFP) signal, *P. betle* was the most effective extract at inhibiting parasite growth in human foreskin fibroblast cells (IC_{50} of GFP-expressing *N. caninum* parasites, 22.1 μg/ml). The *P. betle* extract, at 25 μg per ml, inhibited parasite invasion into host cells. Furthermore, in two independent experiments, treating *N.* caninum-infected mice with the *P. betle* extract for 7 days post-infection increased their survival. In trial one, the anti-*N. caninum* effects of the *P. betle* extract reduced the mouse clinical scores for 30 days post-infection (dpi). The survival rate of the mice treated with 400 mg/kg was 100% compared with 66.6% for those treated with 100 mg/kg and the non-treated controls. In trial two, treating the infected mice with the *P. betle* extract increased their survival at 50 dpi. All mice in the non-treatment group died; however, the survival rates of the 400 mg/kg-treated and 100 mg/kg-treated mice were 83.3% and 33.3%, respectively. Also, a trend towards a reduced parasite burden was noted in the brains of the *P. betle* extract-treated mice, compared with the control mice. Therefore *P. betle* extract has potential as a medicinal plant for treating neosporosis.

Keywords: *Neospora caninum*, neosporosis, *Piper betle*, *Piper nigrum*, *Piper sarmentosum*, ethanol extract

1. Introduction

The apicomplexan parasite *Neospora caninum* is an intracellular protozoan and the causative agent of neosporosis [1]. *N. caninum* is distributed worldwide and has been identified in livestock, companion animals, and wildlife [2,3]. Neosporosis causes economic losses in the livestock industry in many countries, and poses a particular risk to successful reproduction in these animals, as it is known to induce abortion and stillbirth [4–6]. In canine neosporosis, the neurological signs include meningoencephalitis, polymyositis, and polyradiculoneuritis [3,7]. *N. caninum* is transmitted vertically and horizontally. Vertical transmission involves the parasites passing through the placenta, while horizontal transmission involves ingestion of the oocysts shed by the definitive host (canines) via contaminated food or drinking water [1].

To decrease the economic impact of the disease, control strategies include the culling of seropositive animals, prohibiting the breeding of the offspring of seropositive animals, vaccination of seronegative animals, and the chemotherapeutic treatment of infected animals [8,9]. Previous studies have shown the efficacy of chemotherapy and the use of natural treatments such as anti-coccidial drugs [10,11], Chinese herbs [12], sulfadiazine and amprolium [13], ponazuril [14], and artemisinin and its derivative, artemisone [12,15]. Currently, there are no vaccines or chemotherapeutic treatments available that are safe for use on food-producing animals because of the long duration of treatment for the livestock [1,16]. Therefore, identifying natural compounds with anti-*Neospora* activities should be encouraged [8].

Piperaceae (pepper) plants are found in tropical areas including Thailand [17,18]. They are reported to have antibacterial, antioxidant, gastro-protective, and anticancer properties [19]. Additionally, the anti-protozoa effect of these plants against *Leishmania* [20], malaria

parasites [21,22] and *Toxoplasma* [23] has been reported. The effects of piperaceae plants on *Neospora* activity is currently unknown; hence, 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 *N. caninum* infections *in vitro* and *in vivo*.

2. Materials and methods

2.1 Animals, parasites and cell cultures

Six- to eight-week-old female BALB/c mice were obtained from Clea Japan Inc. (Tokyo, Japan). The mice were housed at six per cage and were maintained 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*. This study was conducted in strict accordance with the 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, Obihiro University of Agriculture and Veterinary Medicine (permit numbers 27–30, 28–46). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering.

The *N. caninum* (NC1) parasite and its green fluorescent protein (GFP)-expressing recombinant, (NC-GFP) [24], were propagated in African green monkey kidney (Vero) cells and cultured in Eagle's minimum essential medium (EMEM; Sigma, St. Louis, MO, USA). Subsequently, the cells were supplemented with 8% heat-inactivated fetal bovine serum (FBS), 100 μg/ml penicillin, and 10 mg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Tachyzoites were purified from the infected Vero cells by washing the cells in ice-cold

phosphate-buffered saline (PBS), the final pellet was resuspended in ice-cold PBS, and then passed through a 27-gauge needle syringe three times. The tachyzoites were subjected to filtration through a 5.0-μm pore filter (Millipore, Bedford, MA, USA) to remove the host cell debris, washed twice with 10 ml of PBS, and centrifuged at 1,500 × *g* for 10 min. The tachyzoites were filtered again to obtain a pure preparation, and parasite numbers were counted on a hemacytometer. Human foreskin fibroblast (HFF) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FBS, 100 μg/ml penicillin, and 10 mg/ml streptomycin at 37°C in a 5 % CO_2 atmosphere.

2.2 Plant materials

Fresh leaves from *P. betle* L. and *P. sarmentosum* Roxb. were purchased from Don Wai floating market in Nakhon Pathom Province (central Thailand). The dried seeds of *P. nigrum* L. were purchased from herbal drug stores in Thailand. Although *P. nigrum* grows in all regions [25], most of the plants grow in northeastern Thailand. All plant materials were identified and given serial numbers by the Faculty of Pharmacy, Mahidol University, Thailand. The serials numbers for *P. betle* L., *P. nigrum* L., and *P. sarmentosum* Roxb. plants are PBM05160, PBM05159, PBM05161, respectively. The plant constituents were extracted with 97% ethanol (Sigma-Aldrich, St. Louis, MO, USA) at room temperature (RT) for 3 days. After filtering the solution, the solvent was evaporated with a rotary evaporator (Rotavapor R-200/205; BÜCHI, Flawil, Switzerland) under reduced pressure, and lyophilized to dryness using a freeze dry vacuum system (Labconco, Kansas City, MO USA). The final crude extracts were dissolved in dimethyl sulfoxide at 100 mg/ml and maintained at –30°C.

2.3 Parasite growth assay based on GFP signal

HFF cell suspensions (1×10^5 cells/ml in DMEM supplemented with 10% FBS) were plated at 100 µl/well in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere until the cells reached 100% confluence. NC-GFP (5×10^4 tachyzoites per well) was added to each well for 4 h and the extracellular parasites were washed away. Then, each extract, at final concentrations of 1, 5, 10, 25, 50, and 100 µg/ml (with 100 µl/well of media), was added to the cells for 72 h. Sulfadiazine (1 mg/ml; Sigma) and the control media were used as positive and negative controls, respectively. The fluorescence intensity of the parasites was measured using a fluorescence microplate reader (SH-900; Corona Electric Co., Ibaraki, Japan). The correlation coefficient between the fluorescence intensity of NC-GFP and the number of parasites (a times-two serial dilution ranging from 1,000,000 to 7812.5 parasites) was calculated using the Pearson correlation coefficient and a positive correlation was confirmed [26]. The growth inhibition percentage for NC-GFP was calculated using the fluorescence intensity of NC-GFP according to a previous report [23]. The half maximal inhibitory concentration (IC_{50}), as based on three independent experiments performed together, was calculated using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA).

2.4 Indirect fluorescent antibody test (IFAT)

Vero cells were plated at 1×10^5 cells/well in 12-well plates with round glass coverslips. At 24 h after parasite inoculation, the coverslips were collected and washed twice with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++). Then, the infected cells were fixed overnight at 4°C with 3% paraformaldehyde in PBS++. After washing twice with PBS++, the infected cells were permeabilized with 0.3% Triton X-100 in PBS++ for 5–7 min at RT. After washing twice with PBS++, the coverslips were incubated with 3% bovine serum albumin (BSA) in PBS++ at RT for 30 min. After washing once with PBS++, the coverslips were incubated with an anti-NcSRS2 mouse monoclonal antibody (clone 1B8) [27] diluted 1:100

in 3 % BSA in PBS++for 1 h at RT. After washing three times with PBS++ over a 15 min period, 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++. The nuclear DNA was labeled with Hoechst 33342 (1:10,000 dilution; Thermo Fisher Scientific Inc., Waltham, MA, USA) for 30 min at RT. After mounting the coverslips on a glass slide coated with Mowiol (Calbiochem, San Diego, CA, USA), the slides were examined using an All-in-One Fluorescence Microscope (BZ-9000; Keyence, Tokyo, Japan).

2.5 Invasion assay

The purified extracellular NC-GFP tachyzoites (1×10^6) were pretreated with one of the three piperaceae extracts (25 μg/ml), sulfadiazine (1 mg/ml), or media alone for 1 h at 37°C. The pretreated parasites were then allowed to infect Vero cells in a 12-well plate at a ratio of 10:1 parasites per host cell. The extracellular parasites were washed away after 2 h and EMEM, supplemented with 8% FBS, was added. The infection rates of the *N. caninum* tachyzoites were determined at 24 h post-infection by IFAT, and the NcSRS2-positive Vero cell numbers were counted by randomly selecting 100 Vero cells. The percentage inhibition of infection was expressed as follows: [(average infection rate after treatment with medium alone − infection rate after treatment with either of the extracts) ÷ mean infection rate after treatment with the medium alone] × 100.

2.6 Parasite replication assay

Vero cells, plated at 1 ml/well in 12-well plates (cell suspensions 1×10^5 cells/ml in EMEM supplemented with 8 % FBS), were infected with the purified NC-GFP tachyzoites (2×10^5). The extracellular parasites were washed away at 2 h post-infection, and EMEM, supplemented with 8 % FBS, was added in the presence of either one of the three piperaceae

extracts (25 μg/ml), sulfadiazine (1 mg/ml), or media alone. To determine the inhibition of *N. caninum* tachyzoite replication in Vero cells, the size distribution of parasitophorous vacuoles (PV), expressed as a percentage, was determined by counting the number of parasites containing 1, 2, 4, 8, or 16 parasites per vacuole under a fluorescence microscope (for a total of 100 randomly selected vacuoles) at 24 h post-infection (post-treatment with the extract) based on the NcSRS2 signal measured by IFAT, as described above.

2.7 Effects of the piperaceae extracts on N. caninum infections in vivo

The all mice were intraperitoneally inoculated with freshly purified *N. caninum* (NC1 strain, 1×10^6 tachyzoites/mouse). At 24 h post-infection, the mice were intraperitoneally injected with the *P. betle* extract at 100 and 400 mg/kg/24 h or PBS (n = 6 for each experiment) for 7 days. In the first trial, the mice were observed daily for 30 days post-infection (dpi). For the second trial, the effect of each treatment was monitored for 50 dpi. Daily observations of body weight, morbidity, mortality, and clinical signs were scored and recorded. The scores varied from 0 (no signs) to 10 (all signs). The clinical signs recorded included febrile response/physical appearance, behavior/movement, neurological signs, and dehydration/loss of appetite. Most of the signs recorded were assessed by the criteria used in a previous study [26]. At 30 and 50 dpi, the brains from the surviving mice were collected to determine the parasite burden by quantitative PCR.

2.8 Quantitative PCR for calculating parasite numbers based on the detection of N. caninum DNA

Mouse brain tissue was lysed by adding 10 volumes of extraction buffer (0.1 M Tris-HCl pH 9.0, 1% SDS, 0.1 M NaCl, 1 mM EDTA) and 1 mg/ml of proteinase K at 50°C. DNA was extracted from the tissues using phenol-chloroform extraction methodology and the purified

DNA was precipitated with ethanol. The DNA concentration was adjusted to 25 ng per μl, after which it was used as the PCR template. Parasite burden counts were determined by real-time quantitative PCR, using specifically targeted parasite DNA (Nc5) [28]. The PCR process was performed using an ABI prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), and PCR amplification was monitored using the SYBR Green method (Applied Biosystems). A standard curve was constructed with ten-fold serial dilutions of *N. caninum* DNA extracted from 1×10^5 parasites. The curve ranged from 0.01 to 10,000 parasites. Parasite numbers were calculated by plotting the R values corresponding to 0.962 on a standard curve.

2.9 Statistical analysis

The results were generated and evaluated using GraphPad Prism software 5 (GraphPad Software Inc., La Jolla, CA, USA). The group comparisons were analyzed using a one- or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons procedure. Survival curves were generated using the Kaplan–Meier method, and statistical comparisons between the curves were made using the log-rank method. A P value of < 0.05 was considered statistically significant.

3. Results

3.1 Effect of each piperaceae extract on N. caninum growth in vitro

To determine the anti-*Neospora* effect of each piperaceae extract and sulfadiazine *in vitro*, we examined the fluorescence intensity of NC-GFP in the presence or absence of the extracts. At 72 h post-treatment, the *P. betle* extract inhibited NC-GFP growth at an IC_{50} value of 22.1

μg/ml (Fig. 1A, B). At 50 μg/ml, the *P. nigrum* and *P. sarmentosum* extracts had no effect on NC-GFP growth (Fig. 1A). The IC_{50} for sulfadiazine on NC-GFP was 771.1 μg/ml (Fig. 1C).

3.2 Effects of the piperaceae extracts on extracellular and intracellular N. caninum in vitro

Pretreatment of the parasites with the *P. betle* extract resulted in 100% inhibition of the infection, whereas pretreatment with *P. sarmentosum* or *P. nigrum* extracts resulted in 77.2 ± 7.8% and 52.3 ± 6.7% inhibition, respectively (Fig. 2A). The piperaceae extracts inhibited the parasite infection as compared with the 1 mg/ml sulfadiazine pretreatment (29.5 ± 7.8%) ($P < 0.05$) (Fig. 2A). The GFP signal from the parasites treated with the *P. betle* extract was not identified, indicating that the extract destroyed the parasite cell membrane (Fig. 2B).

To test the effect of each piperaceae extract on parasite replication, NC-GFP-infected Vero cells were treated with 25 μg/ml of either extract (Fig. 3). Only one and two NC-GFP tachyzoites per PV were observed in the cells treated with the *P. betle* extract at 24 h post-infection ($P < 0.05$). Treatment with the *P. sarmentosum* extract produced only slight inhibition of parasite replication, and the proportion of four parasites per PV decreased when compared with the no treatment control ($P < 0.05$).

3.3 Effect of the P. betle extract on N. caninum infection in vivo

Because the *P. betle* extract exhibited anti-*Neospora* activity *in vitro*, the effects of this extract on *Neospora*-infected mice were evaluated based on the subacute (30 dpi) and chronic infection stages (50 dpi). For the first trial, the clinical scores and survival rates of the mice were monitored for 30 dpi (Fig. 4). Clinical signs in the mice were observed from 4 dpi for all the experimental groups; however, treatment with *P. betle* extract at 400 and 100 mg/kg limited the increase of the clinical scores from 6, 7, and 20 to 30 dpi and from 26 to 30 dpi, respectively, compared with the PBS control group (Fig. 4A). The survival rates of the

infected mice at 30 dpi were 100% for those treated with 400 mg/kg, and 66.6% for those treated with 100 mg/kg of the *P. betle* extract and for the PBS-injected group also (Fig. 4B); however, there was no significant difference between the experimental groups ($P > 0.05$). Additionally, *N. caninum* DNA was detected in the brain tissue of all the mouse groups (Fig. 4C).

The effects of the *P. betle* extract were also evaluated at 50 dpi (Fig. 5). Compared with the PBS-injected group, treatment with the *P. betle* extract at 400 and 100 mg/kg limited the increase of the clinical score from 7, 8, and 21 to 29 dpi, and from 7, 8, and 27 to 29 dpi, respectively ($P < 0.05$) (Fig. 5A). The survival rates of the mice treated with 400 and 100 mg/kg of the *P. betle* extract were 83.3 % ($P = 0.0013$) and 33.3 % ($P = 0.27$), respectively, while the PBS-injected mice died within 30 dpi (Fig. 5B). Furthermore, the number of parasites in the brain tissues of the mice treated with the *P. betle* extract showed a tendency to decrease compared with the PBS-injected mice (Fig. 5C).

4. Discussion

N. caninum causes neuromuscular paralysis in dogs and reproductive failure in cattle, thereby contributing to economic losses worldwide [1,4,5]. Chemotherapy for seropositive animals is one possible treatment option, but the treatment needs to be safe and effective, and must also exhibit limited toxicity, particularly when used in food-producing animals [8,29]. Previous studies have reported on various compounds with effective anti-parasite activities including a triazinone derivative, thiazolides, sesquiterpene lactone, dicationic arylimidamide, substituted pyrazolopyrimidines, and naphtoquinone, all of which have been tested *in vitro* and *in vivo* [8]. Herbal medicine and natural compounds both have the ability to inhibit growth in apicomplexan parasites [30, 31]. Artemisinin and its derivatives [15], and *Torilis*

japonica and *Sophora flavescens* [32] can also inhibit *Neospora* infections. Therefore, herbal medicine and natural compounds might be alternative methods for neosporosis treatment. To date, few studies have investigated treatment with herbs and natural compounds for *N. caninum* infections. In the present study, we found that *P. betle* extract was effective at inhibiting *N. caninum in vitro* and *in vivo*.

In our previous study, the anti-*Toxoplasma* properties of piperaceae extracts were confirmed both *in vitro* and *in vivo* [23]. Therefore, further study on the effects of piperaceae plants will provide an opportunity to discover new phytochemical compounds that have potential anti-*Neospora* properties. In the present study, pretreating *N. caninum* tachyzoites with *P. betle* extract completely inhibited parasite invasion of Vero cells. Furthermore, the *P. betle* extract reduced proliferation of the intracellular parasites via cell membrane disruption. Extracts of *P. sarmentosum* and *P. nigrum* significantly inhibited parasite invasion compared with sulfadiazine pretreatment; however, these extracts had no effect on the intracellular parasites. These results are consistent with those reported in the previous *Toxoplasma* study referred to above [23]. The IC_{50} value of the *P. betle* extract for intracellular *N. caninum* was 22.1 μg/ml, while the values for *Toxoplasma gondii* RH and PLK strains were 23.2 μg/ml and 21.4 μg/ml, respectively [23]. These results suggest that the phytochemical compounds in the piperaceae plants target the same metabolic pathway in *N. caninum* and *T. gondii*.

In the present study, treating the *Neospora*-infected mice with the *P. betle* extract increased their survival rates, particularly when the highest concentration (400 mg/kg) was used. The extracts reduced the clinical signs of infection during the acute and subacute stages. When analyzing the cytotoxicity of the *P. betle* extract, our previous study found the IC_{50} of this extract against HFF cells was 180.2 μg/ml [23]. *In vivo*, oral administration of the *P. betle* ethanol extract at 1,500 mg/kg/day for 40 consecutive days showed no significant side effects in cross-bred male albino rats [33]. Therefore, is seems likely that *P. betle* extract is safe to

use as a treatment regimen and is worth investigating further for the treatment of neosporosis. Although the IC_{50} of sulfadiazine (an anti-*Toxoplasma* drug) against the growth of *T. gondii* RH and PLK strains was 99.4 μg/ml and 22.3 μg/ml, respectively [23], in the present study, its IC_{50} against *N. caninum* growth was 771.1 μg/ml. This indicates that sulfadiazine treatment may be less effective against infection with *N. caninum*. Hence, phytochemical compounds in the *P. betle* extract merit further investigation as potential drugs for controlling *N. caninum* infections.

Because the *P. betle* extract has anti-oxidant, anti-inflammatory, and immunomodulatory properties [19], it might cure the *N. caninum*-infected mice by reducing the severe inflammation that follows an *N. caninum* infection. Additionally, the direct parasite killing effects of the *P. betle* extract will have contributed to increased survival rates in the infected mice and decreased the parasite burden in the brain. However, the mechanism by which the parasite is killed by the *P. betle* extract is unknown. The main phytochemicals in *P. betle* leaves are alkaloids, terpenes, anthraquinones, flavonoids, tannins, saponins, and steroids [22]. Phytochemical compounds such as alkaloids and flavonoids have antiplasmodial properties [22]. Alkaloids induce apoptosis in *Trypanosoma brucei,* as evidenced by DNA fragmentation and a change in the mitochondrial membrane [34]. Treatment with terpenes (e.g. farnesol, nerolidol, limonene and linalool) arrest the development of *Plasmodium falciparum* because these treatments inhibit the biosynthesis of several intermediates and end products of the isoprenoid pathway, which are important for the synthesis of steroids, cholesterol, retinoids, carotenoids, and ubiquinones [35]. Flavonoids such as quercetin and genistein, and polyphenolic compounds have many cellular targets in *Toxoplasma*. Flavonoids inhibit the infectivity, proliferation and protein kinases of *T. gondii* [36]. Quercetin prevents bradyzoite induction by inhibiting expression of the *Toxoplasma* heat shock proteins involved in bradyzoite development [37]. In addition, genistein treatment

reduces the infectivity of *T. gondii* by interfering with MAP kinase activation pathways [38]. Thus, the alkaloids, terpenes and flavonoids in *P. betle* have potential anti-*Neospora* activities.

In the present study, we found that the *P. betle* extract effectively inhibited *N. caninum in vitro* and *in vivo*. These results indicate that the active ingredients in the *P. betle* extract may have the potential to treat neosporosis. In future studies, it would be beneficial to separate the extract into its chemical components to identify the active phytochemicals with anti-*Neospora* properties.

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Figure legends

Fig. 1. Anti-*Neospora* properties of piperaceae plant extracts *in vitro*. (A) Representative images of *N. caninum* NC-GFP-infected human foreskin fibroblast cells treated with either sulfadiazine (1 mg/ml), *P. betle* (50 μg/ml), *P. nigrum* (50 μg/ml), or *P. sarmentosum* (50 μg/ml) for 72 h. (B) The IC_{50} values of the *P. betle* extract on NC-GFP. (C) The IC_{50} of sulfadiazine on NC-GFP.

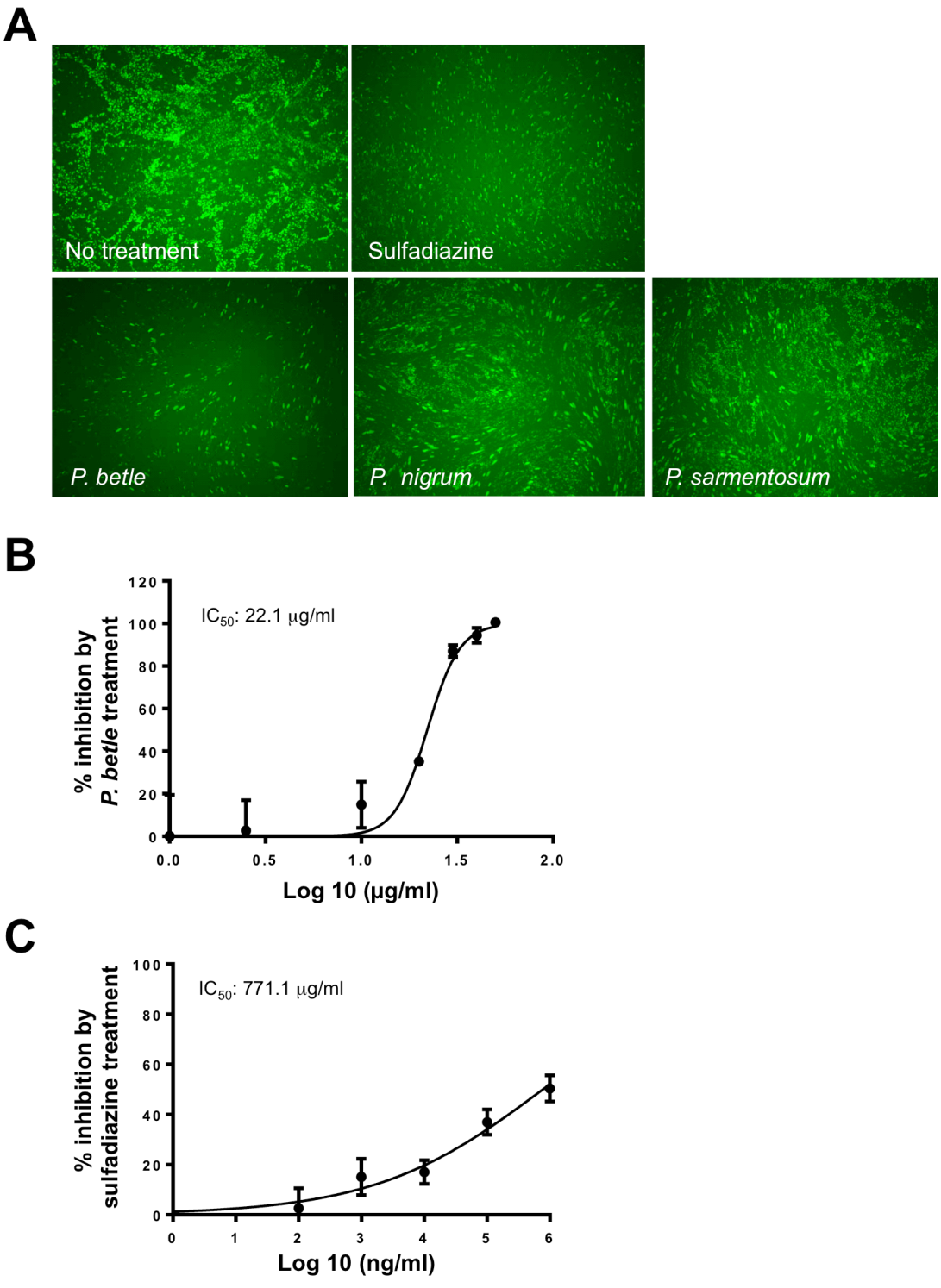
A
No treatment
Sulfadiazine
P. betle
P. nigrum
P. sarmentosum
B
% inhibition by P. betle treatment
IC50: 22.1 μg/ml
Log 10 (μg/ml)
C
% inhibition by sulfadiazine treatment
IC50: 771.1 μg/ml
Log 10 (ng/ml)

Fig. 1

Fig. 2. Effects of the three piperaceae extracts and sulfadiazine on extracellular *N. caninum*. NC-GFP parasites were pre-treated with either *P. betle*, *P. nigrum*, *P. sarmentosum* (25 μg/ml) or sulfadiazine (1 mg/ml) for 1 h, and were then used to infect Vero cells. After 24 h, the infected cells were analyzed by IFAT to measure the infection rates. (A) The percentage inhibition of infection for NC-GFP was measured by counting the number of NcSRS2-positive Vero cells per 100 Vero cells. Each bar represents the mean ± SD of three wells per group. 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 ($P < 0.05$). (B) Representative images of NC-GFP-infected Vero cells. NcSRS2, red; GFP, green; nuclei, blue.

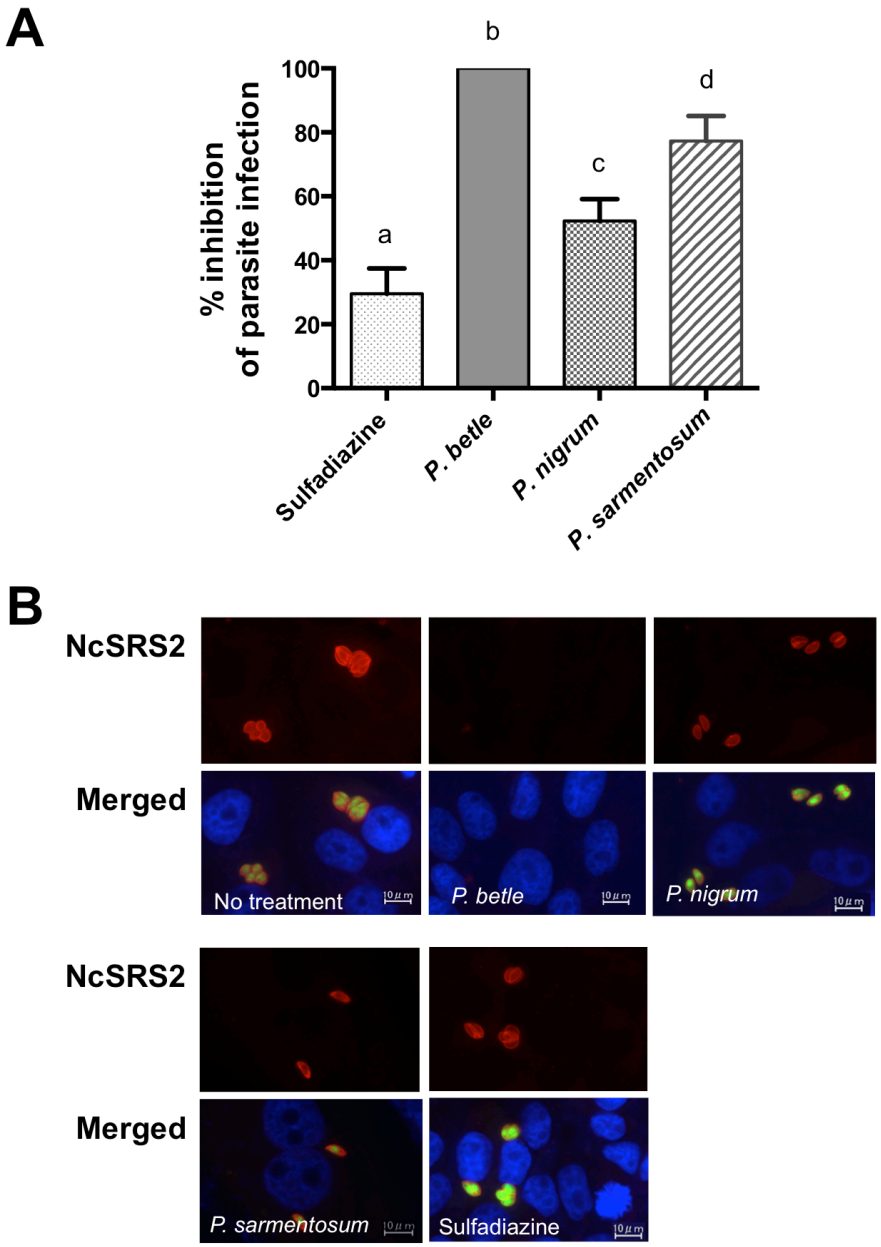
A
b
d
c
a
100
80
60
40
20
0
% inhibition of parasite infection
Sulfadiazine
P. betle
P. nigrum
P. sarmentosum
B
NcSRS2
Merged
No treatment
P. betle
P. nigrum
NcSRS2
Merged
P. sarmentosum
Sulfadiazine

Fig. 2

Fig 3. Effects of the three piperaceae extracts on intracellular *N. caninum*. NC-GFP-infected Vero cells were treated with either *P. betle*, *P. nigrum*, *P. sarmentosum* (25 μg/ml) or sulfadiazine (1 mg/ml) for 24 h, and subsequently analyzed using IFAT to measure the parasitophorous vacuole (PV) sizes. (A) The number of parasites in PVs was measured by counting the number of NcSRS2-positive parasites per PV. Each bar represents the mean ± SD of three wells per group. Data are representative from two independent experiments with similar results. The different letters indicate statistically significant differences in the number of parasites in PVs as determined by two-way ANOVA plus Tukey–Kramer post-hoc analysis ($P < 0.05$). (B) Representative images of NC-GFP-infected Vero cells. NcSRS2, red; GFP, green; nuclei, blue.

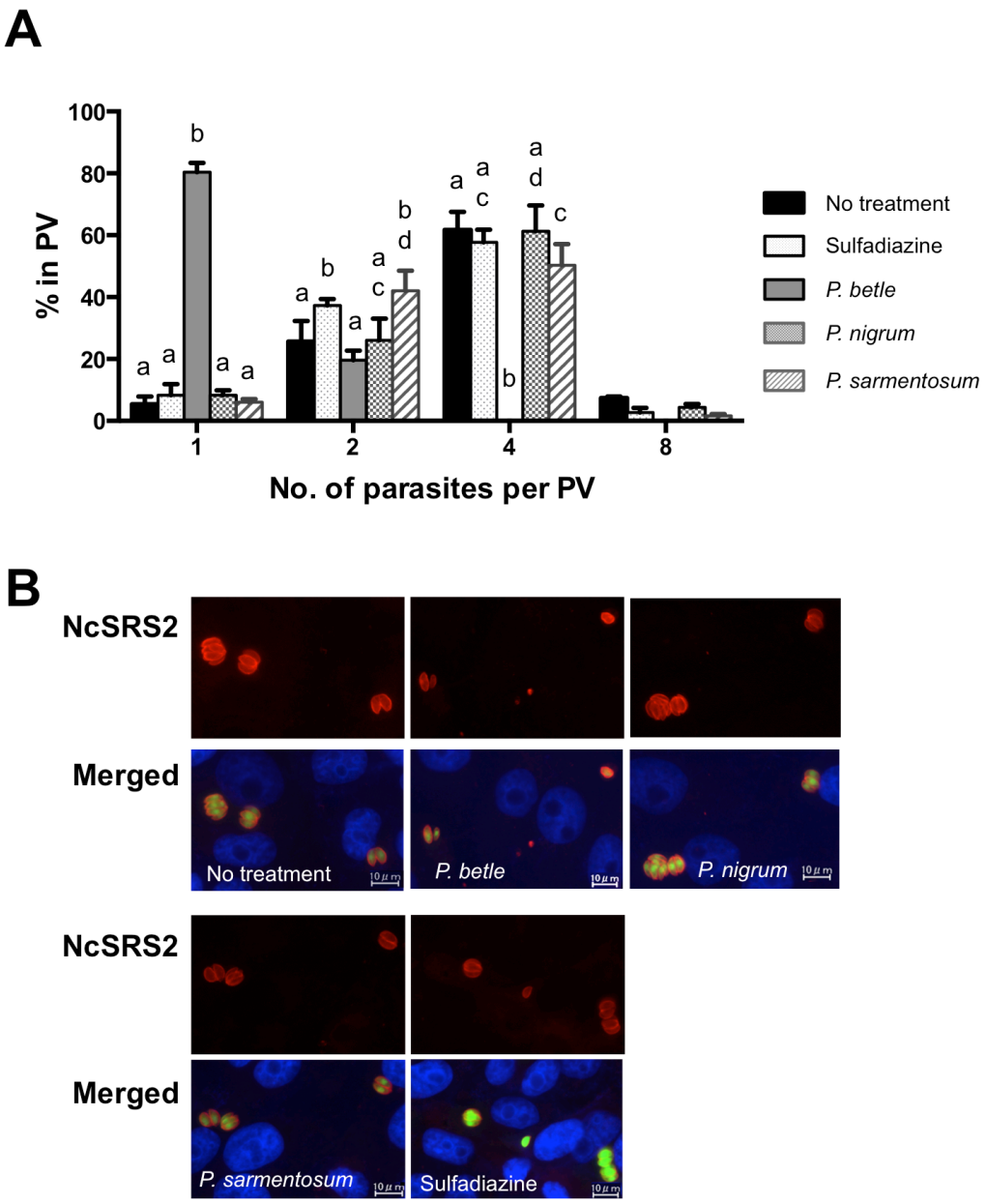
A
100
80
60
40
20
0
% in PV
a a b a a
a b a a c b d
a a c b a d c
1
2
4
8
No. of parasites per PV
No treatment
Sulfadiazine
P. betle
P. nigrum
P. sarmentosum
B
NcSRS2
Merged
No treatment
P. betle
P. nigrum
NcSRS2
Merged
P. sarmentosum
Sulfadiazine

Fig. 3

Fig 4. Clinical scores and survival rates for the 30 day post-infection period, and parasite burdens in mouse brain tissue. Mice were intraperitoneally injected with *P. betle* extracts at 100 and 400 mg/kg/day or PBS from 1 to 7 days post-infection (dpi), with 1×10^6 NC1 tachyzoites. Clinical scores and survival rates were monitored for 30 dpi. 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 (n = 6 per group). (A) The clinical scores were analyzed using a two-way ANOVA and 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, $P < 0.05$). (B) Survival curves were generated using the Kaplan–Meier method. According to the log-rank test, the differences between the PBS and *P. betle* extract treatment groups were not significant. (C) Parasite burdens in the brain tissues of the surviving mice. The parasite counts in 50 ng of tissue DNA per individual (symbols) and the mean levels (horizontal lines) are indicated. The mean parasite counts were analyzed using a one-way ANOVA and Tukey–Kramer post-hoc analysis; however, there were no significant differences in the values among the experimental groups.

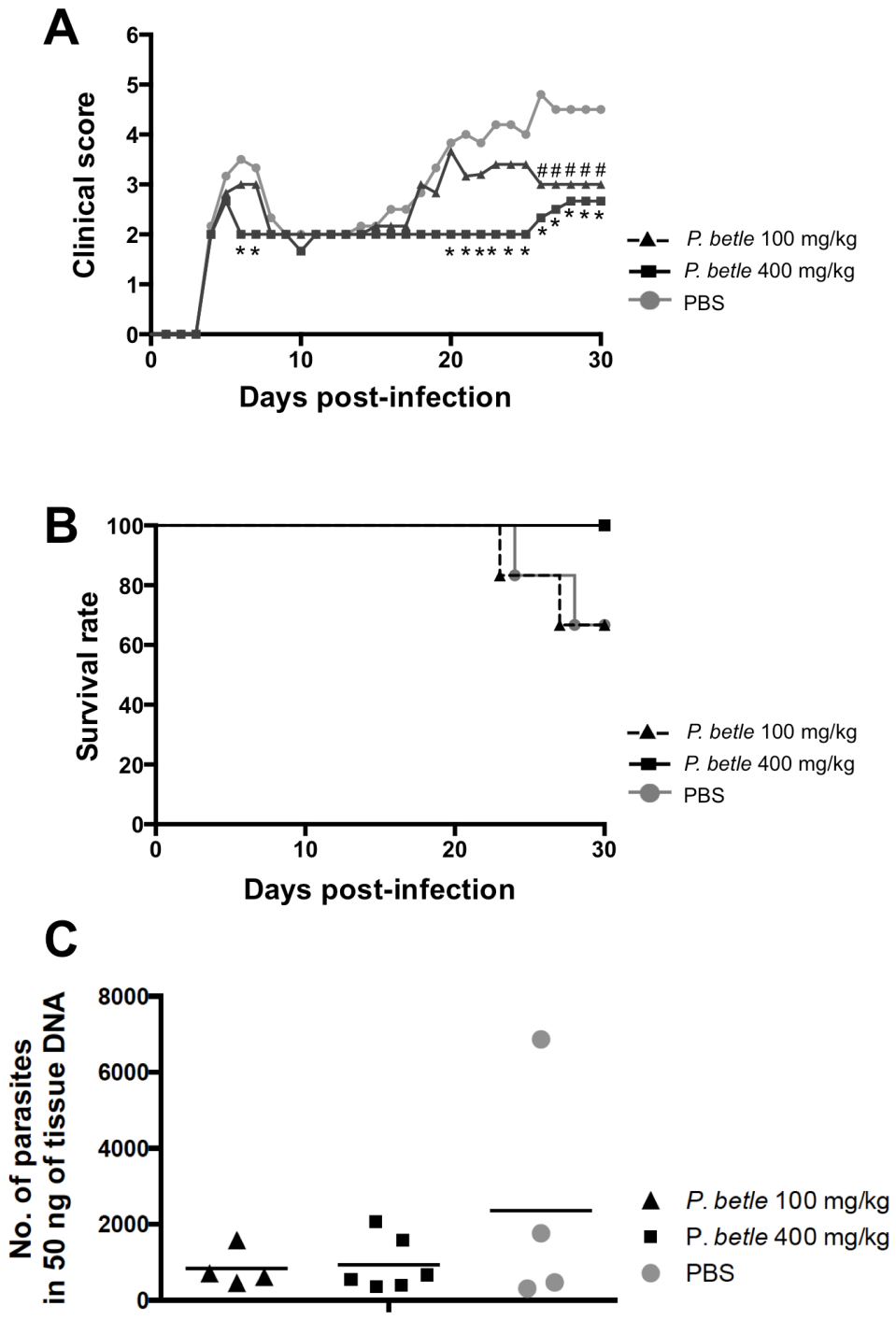
A
Clinical score
Days post-infection
P. betle 100 mg/kg
P. betle 400 mg/kg
PBS
B
Survival rate
Days post-infection
P. betle 100 mg/kg
P. betle 400 mg/kg
PBS
C
No. of parasites in 50 ng of tissue DNA
P. betle 100 mg/kg
P. betle 400 mg/kg
PBS

Fig. 4

Fig 5. Clinical scores and survival rates for the 50 day post-infection period, and parasite burdens in mouse brain tissue. Mice were intraperitoneally injected with *P. betle* extracts at 100 and 400 mg/kg/day or PBS from 1 to 7 days post-infection (dpi), with 1×10^6 NC1 tachyzoites. Clinical scores and survival rates were monitored for 50 dpi. 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 (n = 6 per group). (A) The clinical scores represent the mean total values for all mice used in this study. Data represent the mean values of all the mice. Because all the mice died in the PBS-injected group within 30 days post-infection, the clinical scores were analyzed for 29 days using a two-way ANOVA and 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, $P < 0.05$). (B) Survival curves were generated using 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 400 mg/kg/day, $P < 0.001$). (C) Parasite burden in mouse brain tissue. The brains from the dead and surviving mice were collected at the end of the observation period (50 dpi) for parasite detection. Each symbol represents the parasite count and the red symbol is the brain tissue collected from the dead mice, but some samples could not be collected. The mean parasite counts were calculated using one-way ANOVA and Tukey–Kramer post-hoc analysis, but no significant differences in the values were observed among the experimental groups.

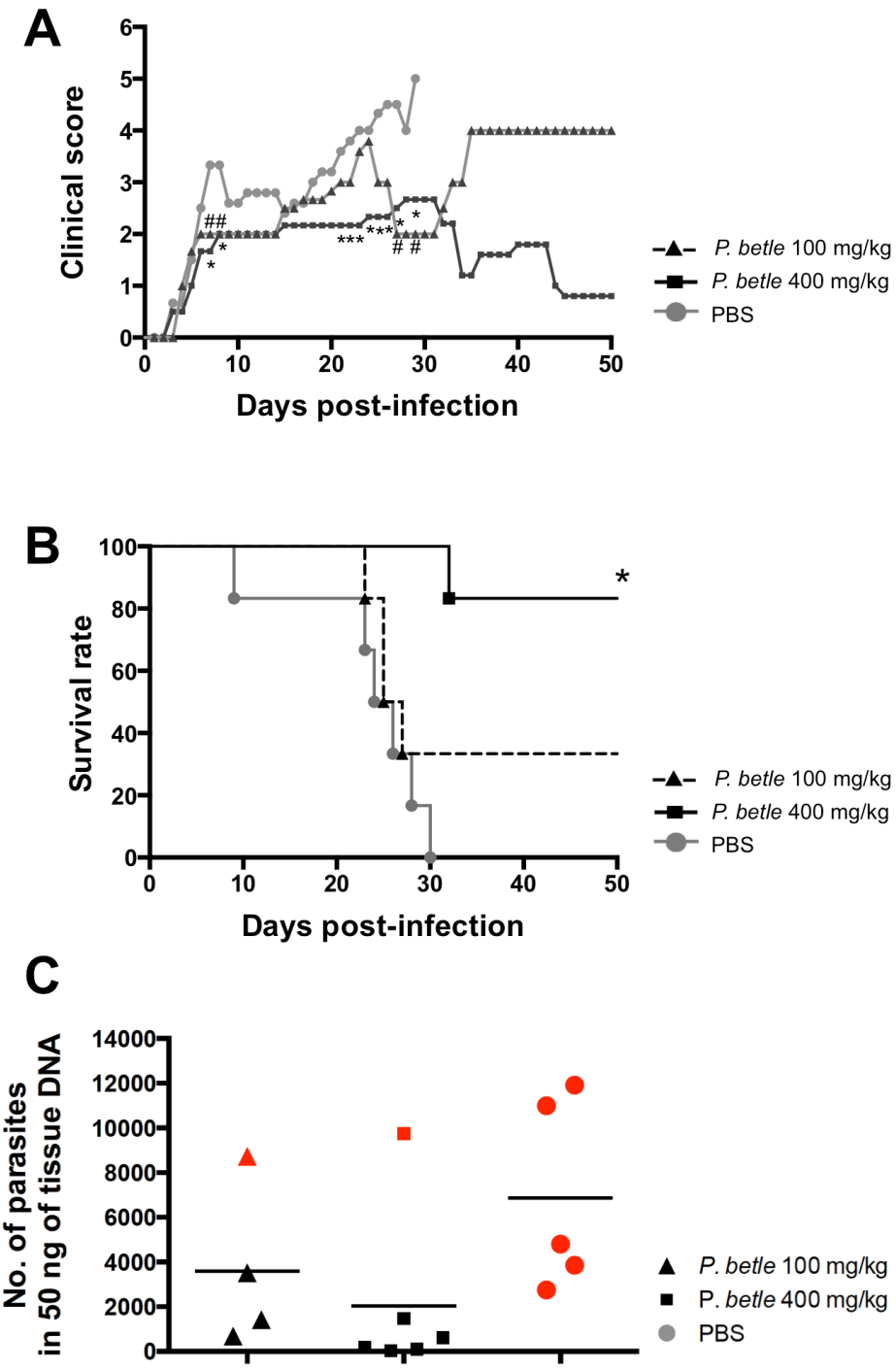
A
Clinical score
Days post-infection
P. betle 100 mg/kg
P. betle 400 mg/kg
PBS
B
Survival rate
Days post-infection
P. betle 100 mg/kg
P. betle 400 mg/kg
PBS
C
No. of parasites in 50 ng of tissue DNA
P. betle 100 mg/kg
P. betle 400 mg/kg
PBS

Fig. 5