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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.

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

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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 1500 ×*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

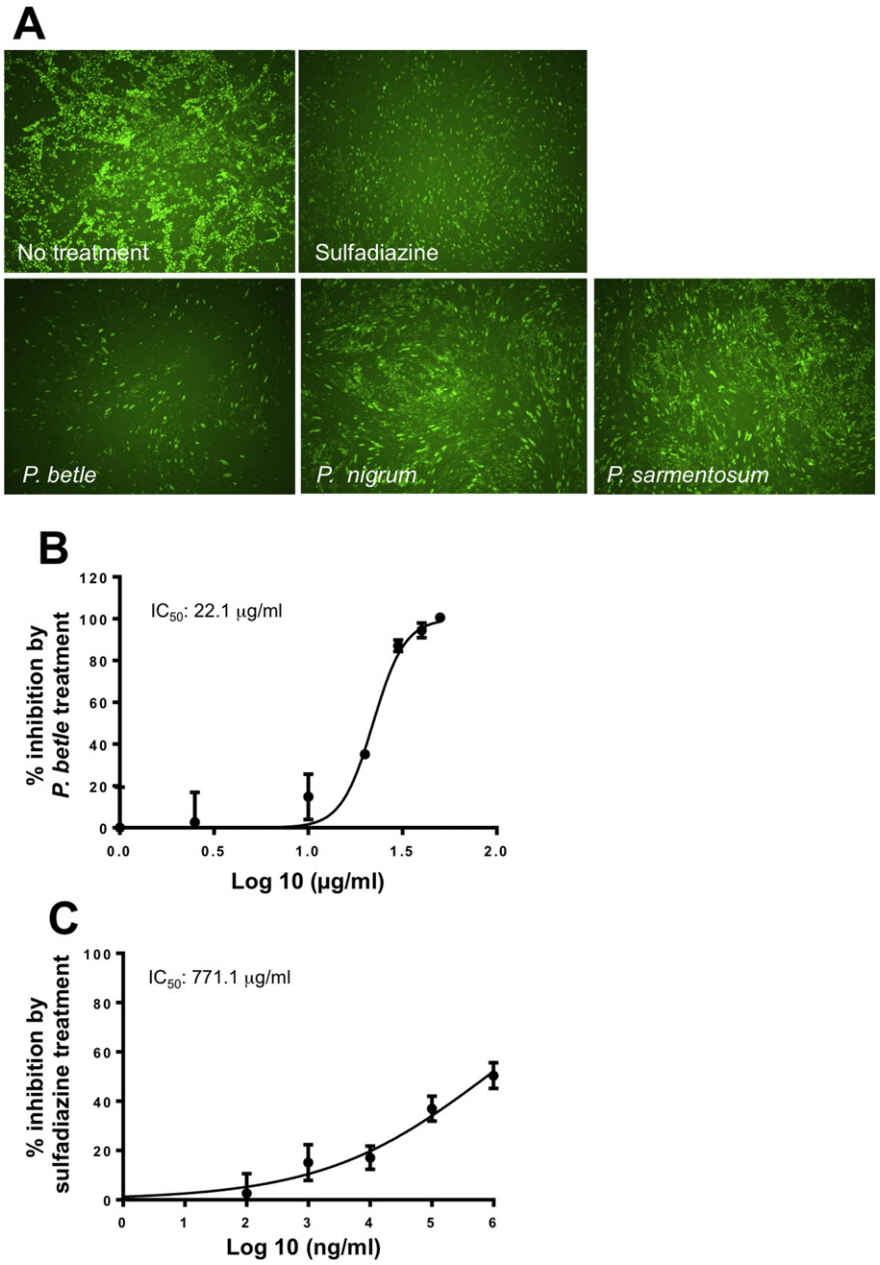

Fig. 1. Anti-*Neospora* properties of piperaceae plant extracts *in vitro*. (A) Representative images of *N. caninum* NC-GFP-infected human foreskin fibroblast cells treated with either sulfadiazine (1 mg/ml), *P. betle* (50 μg/ml), *P. nigrum* (50 μg/ml), or *P. sarmentosum* (50 μg/ml) for 72 h. (B)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