Journal of Apiculture

2019.34(2) : 137-140
DOI: 10.17519/apiculture.2019.06.34.2.137

Original research article

Anti-Parasitic Activity of *Lespedeza cuneata* Extract on Causative Agent of Nosemosis Type C, *Nosema ceranae*

Hyunchan Song, Hyekyung Kim[1] and Ki-Young Kim*

Graduate School of Biotechnology, Kyung Hee University, 1 Seocheon, Giheung-gu, Yongin-si, Gyeonggi-do 17104, Republic of Korea
[1]Department of Industrial Entomology, Korea National College of Agriculture and Fisheries, Jeonju-si, Jeollabuk-do, Republic of Korea

Abstract Although honeybees (*Apis mellifera*) are crucial for maintenance of the ecosystem, population of honeybee has been steadily decreasing due to diseases including nosemosis. Nosemosis is a disease caused by *Nosema ceranae* and is now considered as a major threat to honeybees. *N. ceranae* is a microsporidian that stays in form of spore even before the infection, which makes it harder to control than other pathogens. People are now aware of this parasite, however, cure and preventive candidates for nosemosis are hardly found until today. In this study, *in vitro* experiment of *Lespedeza cuneata* treatment to prevent nosemosis were done using *Trichoplusia ni* cell line, BTI-TN5B1-4. Normal *T. ni* cells exhibited round shape without abnormal size. On the other hand, when *N. ceranae* were treated, cells deteriorated and some cells abnormally enlarged due to *N. ceranae* infection. Interestingly, treatment of *T. ni* cells with *L. cuneate* extract protected abnormal cell shape induced by *N. ceranae* infection to normal shape. Some *N. ceranae* spores were observed outside of the cells. Effective concentration range for *N. ceranae* control were experimented. Lowest concentration which can control nosemosis were 50 μg/mL. When the concentration of *L. cuneata* extract was exceeded 200 μg/mL, cytotoxicity started to show up.

Keywords *Nosema ceranae*, *Lespedeza cuneata*, Nosemosis

INTRODUCTION

Honeybees (*Apis mellifera*) are essential for our ecosystem as pollinators. They help crop and fruit pollinations, and as a result, their economic value was estimated $14.6 billion in 2000 (Morse and Calderone, 2000). Even after those research, their value has not stopped increasing since. However, this valuable insect has been exposed to many pathogens. Not too long ago, their colonies started to disappear even more often without exact cause. This phenomenon was termed colony collapse disorder (CCD) and the term appeared more in literature than before (Evans *et al*., 2009). Although the reason for the CCD is still unknown, nosemosis is considered as one of the plausible cause (Ellis *et al*., 2010).

Nosema ceranae is a microsporidian, a spore-forming fungi, and is causative of nosemosis (Fries, 2010). When *N. ceranae* enters inside of honeybee by ingestion, they infect honeybee midgut cells. After they start to develop inside of honeybees, the metabolism of honeybees changes and they fully grow after 5 days (Mayack and Naug, 2009). Then, 15 days after the infection, honeybees start to die.

There were many trials to find the cure or preventive medicine for nosemosis, however, results were not enough compared to efforts given. Fumagillin were considered

Received 22 February 2019; Revised 29 April 2019; Accepted 3 May 2019
*Corresponding author. E-mail: kiyoung@khu.ac.kr

great anti-parasitic chemical to control nosemosis once, but, recent study revealed that fumagillin may not be a great anti-parasitic compound for *N. ceranae* control and it is now harder to control even more (Fenoy, 2009; Huang, 2013).

Here, we tested *Lespedeza cuneata* extract as an anti -nosemosis solution. We used *Trichoplusia ni* cell line, BTI-TN5B1-4, as an alternative for honeybee, since honeybee cell line are not found still (Fries, 1988). *L. cuneata*-treated cells showed no symptoms of nosemosis even though *N. ceranae* was treated together. Effective concentration ranges of *L. cuneata* were tested where there was no cytotoxicity while great anti-parasitic activity was still found.

MATERIALS AND METHODS

Spore preparation

N. ceranae-infected Honeybees were obtained from Rural Development Administration in Korea. Midgut of honeybees with nosemosis were homogenized in 200 μL of distilled water to obtain *N. ceranae* spores. *N. ceranae* was purified using discontinuous 25%, 50%, 75% and 90% percoll (GE healthcare, USA) (Kim *et al*., 2017). The concentration of spore was calculated using hemocytometer. Spores were diluted with distilled water and set to 10,000 spores/μL.

Extract preparation

L. cuneata were washed with distilled water before whole extraction process. 100 g of *L. cuneata* were extracted using 1 L of 80% ethanol at room temperature for 24 hours in shaking condition. The solvents were evaporated at 50°C using rotary evaporator (EYELA, Japan) which was connected to refrigerated bath circulator (Jeio Tech, Korea).

Insect cell maintenance

BTI-TN5B1-4 were used in this paper. Cells were incubated in express five SFM (Gibco, USA) supplemented with Glutamine (Gibco, USA) at 27°C. Cells were stored in −70°C until use.

N. ceranae and *L. cuneata* treatment

After incubating 2 mL of BTI-TN5B1-4 in 6-well plate for a day, 10^4 spores/mL of *N. ceranae* spores were treated directly to the well except the control. For samples with *L. cuneata* treatment, the extract was treated as concentration doubling from 12.5 μg/mL to 800 μg/mL. To observe infection, *N. ceranae* treated cells were incubated for 5 days after *N. ceranae* treatment.

Microscopy of nosemosis

Microscope image of BTI-TN5B1-4 cells with/without *N. ceranae* and cells with both *N. ceranae* and *L. cuneata* extract were taken by cell imaging system (Thermo, USA). Results were determined by their appearances, and those appearances were used as a standard of effective concentration.

Statistical analysis

In order to determine the reproducibility of the measurements, *L. cuneata* extract treatment on BTI-TN5B1-4 cells was triplicated.

RESULTS

Anti-parasitic activity of *L. cuneata* extract against nosemosis

BTI-TN5B1-4 cells without any treatment exhibited round shape (Fig. 1). There were no cells abnormally enlarged, most of the nucleus of cells were clearly visible. Some of cells showed weirdly lengthened and polygonal shape. However, it is thought to be due to their long growth time, not because of their natural phenotype. Many of BTI-TN5B1-4 cells only with *N. ceranae* showed shrunk cell shape compared to normal cells, and some of them were abnormally enlarged (Fig. 2). Nucleus of those with enlarged shape were not clear and many spores of *N. ceranae* are found inside of the cell. On the other hand, BTI-TN5B1-4 cells with both *N. ceranae* and *L. cuneata* extract showed similar cell shape with untreated control (Fig. 3). Their shape was not much different from normal cells. Most of *N. ceranae* spores were found outside of the cell.

Functional concentration of *L. cuneata* extract against nosemosis

To find the lowest concentration of *L. cuneata* extract which is still effective, the extract was treated at con-

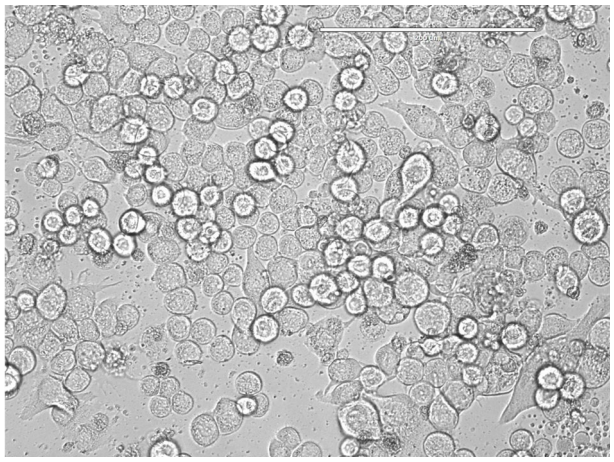

Fig. 1. Microscopic image of BTI-TN5B1-4 cells without any treatment. Cells were circular with only few exceptions.

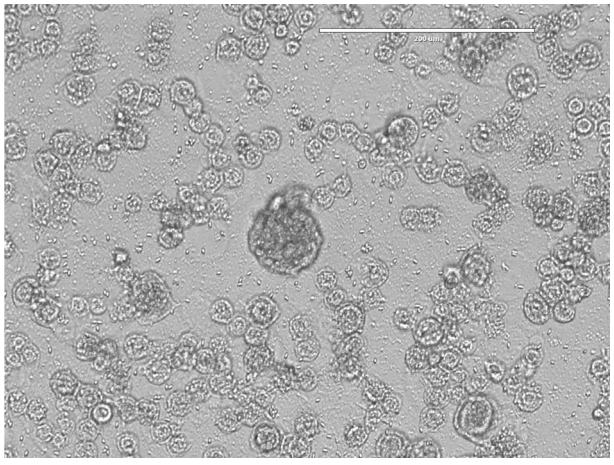

Fig. 2. Microscopic image of BTI-TN5B1-4 cells treated only with *N. ceranae*. Cells were shrunk and some cells were clearly enlarged compared to normal cells.

centration lower than 100 μg/mL. Until 50 μg/mL, *L. cuneata* extract showed the same anti-parasitic activity as 100 μg/mL, but at concentrations lower than 50 μg/mL, it did not show anti-parasitic activity, similar to the ones without it (Fig. 3). They were not different from cells treated only with *N. ceranae* (Fig. 2). In order to find the highest concentration without cytotoxicity, the extract was treated at concentration higher than 100 μg/mL. At 200 μg/mL, the extract still did not show any cytotoxicity and had great anti-parasitic activity. However, at concentration over 200 μg/mL, *L. cuneata* extract start to induce cytotoxic effect on BTI-TN5B1-4 cells (Fig. 4).

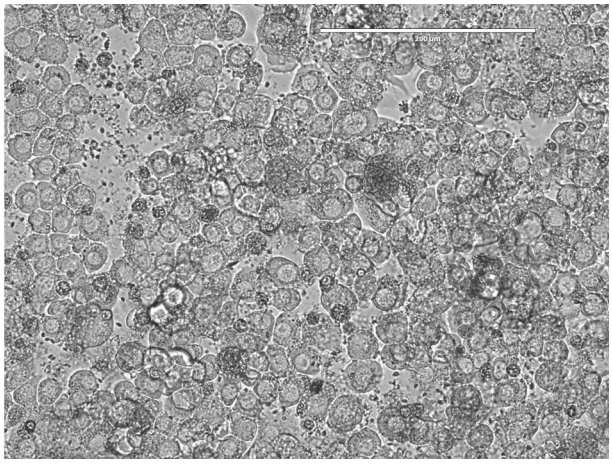

Fig. 3. Microscopic image of BTI-TN5B1-4 cells treated with *N. ceranae* and *L. cuneata* extract. *L. cuneata* extract were treated at concentration of 100 μg/mL. Most of the cells remained uninfected and normal.

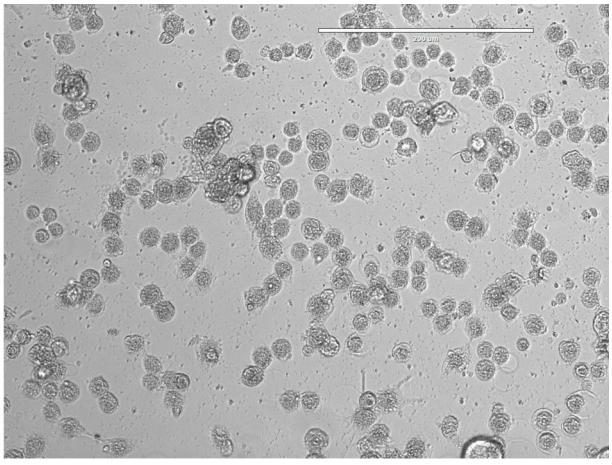

Fig. 4. Microscopic image of BTI-TN5B1-4 cells treated with *N. ceranae* and 400 μg/mL *L. cuneata* extract.

DISCUSSION

Reports emphasizing the effect of nosemosis on our ecosystem has been reported frequently (Klee *et al*., 2007; Hges *et al*., 2010). Since nosemosis was pointed as one cause of CCD, studies finding the cure for *Nosema* spp. are appearing more than before (Ellis *et al*., 2010). However, there are still no definite preventive medicine for it, and honeybees are still staying vulnerable to the disease.

We are now added a candidate for the preventive chemical for nosemosis with *L. cuneata* extract. *L. cuneata*

extract had a great anti-parasitic activity against *N. ceranae*. Surprisingly, cells treated with *L. cuneata* extract seemed to have resistance against *N. ceranae* even after 5 days, which might suggest that there was no initiation of infection when cells were treated with the extract. It was more interesting to see that most of the *N. ceranae* spores could not enter the cell, and stayed around it.

Our results are proposing the appropriate concentration to use *L. cuneata* extract as a preventive treatment for nosemosis. We determined the lowest, but functional concentration of *L. cuneata* extract against nosemosis. At 50 μg/mL, *L. cuneata* extract was still effective, showing no abnormal symptoms like the ones with nosemosis. Unfortunately, under 50 μg/mL, anti-parasitic activity of *L. cuneata* extract was lost. We also determined the highest concentration of *L. cuneata* extract with no cytotoxicity, since too much chemicals may harm the cells. 200 μg/mL was the highest concentration without cytotoxicity, and over that concentration, cells shrunk abnormally in size.

It is important to note that *L. cuneata* is now considered as an invasive plant in over the world (Allred, 2010). It invades habitat of native plants in many countries. Instead of abandoning this invader, it would be economically efficient to use *L. cuneata* as a preventive medicine against nosemosis.

The *in vivo* study of *L. cuneata* on honeybees was not demonstrated in this study. To fully ensure the safety and effectiveness on honeybees, *in vivo* study of *L. cuneata* on honeybees to control nosemosis will be performed in further study.

ACKNOWLEDGEMENTS

This research was supported by the IPET (116094-03-1-SB010).

FOOTNOTES

Financial Disclosure: This research was conducted in the absence of any commercial or financial relationships that could be construed as posing potential conflicts of interest.

LITERATURE CITED

Allred, B. W., S. D. Fuhlendorf, T. A. Monaco and R. E. Will. 2010. Morphological and physiological traits in the success of the invasive plant *Lespedeza cuneata*. Biol. Invasions. 12(4): 739-749.

Ellis, J. D., J. D. Evans and J. Pettis. 2010. Colony losses, managed colony population decline, and Colony Collapse Disorder in the United States. J. Apic. Res. 49(1): 134-136.

Evans, J. D., C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy and J. S. Pettis. 2009. Colony collapse disorder: a descriptive study. PloS ONE. 4(8): e6481.

Fenoy, S., C. Rueda, M. Higes, R. Martín-Hernández and C. Del Aguila. 2009. High-level resistance of *Nosema ceranae,* a parasite of the honeybee, to temperature and desiccation. Appl. Environ. Microbiol. 75(21): 6886-6889.

Fries, I. 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invertebr. Pathol. 103: S73-S79.

Fries, I. 1988. Infectivity and multiplication of *Nosema apis* Z. in the ventriculus of the honey bee. Apidologie. 19(3): 319-328.

Hges, M., R. Martín-Hernández and A. Meana. 2010. *Nosema ceranae* in Europe: an emergent type C nosemosis. Apidologie. 41(3): 375-392.

Huang, W. F., L. F. Solter, P. M. Yau and B. S. Imai. 2013. *Nosema ceranae* escapes fumagillin control in honey bees. PLoS Pathog. 9(3): e1003185.

Kim, D. J., H. G. Yun, I. H. Kim, W. S. Gwak and S. D. Woo. 2017. Efficient Method for the Rapid Purification of *Nosema ceranae* Spores. Mycobiology. 45(3): 204-208.

Klee, J., A. M. Besana, E. Genersch, S. Gisder, A. Nanetti, D. Q. Tam, T. X. Chinh, F. Puerta, J. M. Ruz, P. Kryger, D. Message, F. Hatjina, S. Korpela, I. Fries and R. J. Paxton. 2007. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J. Invertebr. Pathol. 96(1): 1-10.

Mayack, C. and D. Naug. 2009. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100(3): 185-188.

Morse, R. A. and N. W. Calderone. 2000. The value of honey bees as pollinators of US crops in 2000. Bee Culture 128(3): 1-15.