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Original Article

EVALUATION OF ANTIMICROSPORIDIAN ACTIVITY OF PLANT EXTRACTS ON *NOSEMA CERANAE*

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Abstract

Nosemosis is one of the most common protozoan diseases of adult bees (*Apis mellifera*). Nosemosis is caused by two species of microsporidia; *Nosema apis* and *Nosema ceranae*. *Nosema ceranae* is potentially more dangerous because it has the ability to infect multiple cell types, and it is now the predominant microsporidian species in *A. mellifera*. In this study, we identified two anti-nosemosis plants, *Aster scaber* and *Artemisia dubia*, which reduced the spore development of *N. ceranae* in spore-infected cells. The most important aspect of our results was that our treatment was effective at non-toxic concentrations. Anti-nosemosis activities of both plants were revealed in honey bee experiments. Specifically, a mixed extract of both *A. scaber* and *A. dubia* showed stronger activity than treatment with each single extract alone. Although the mechanisms of action of *A. scaber* and *A. dubia* against *N. ceranae* are still unclear, our results suggest new medicaments and therapeutic methods to control *N. ceranae* infection.

Keywords: ***Artemisia dubia, Aster scaber, Nosema cerana***

INTRODUCTION

Honey bees are known for manufacturing and keeping honey, as well as building remarkably large cages using wax. As pollinators, honey bees are important to the environment as well as the food supply (Calderone, 2012). There are many species of honey bees spread across the world and they can be seen in many different locations. The honey bee is a member of the genus *Apis*. The two most important for beekeeping are the western honey bee, *Apis mellifera*, and the eastern honey bee, *Apis cerana*.

The genus Nosema, which belongs to the class Microsporidia, contains more than 150 species including *Nosema ceranae* and *Nosema apis* which cause Nosemosis (Fries, 2010; Higes, Martin, & Meana, 2006). A spore of *N. apis* forms in the midgut epithelium of the honey bee. The sporulation of *N. ceranae* is not restricted to the midgut but may also occur in other tissues, including the alimentary canal, malpighian tubules, hypopharyngeal glands, salivary glands, and fat bodies (Chen & Huang, 2010). N. ceranae is potentially more dangerous because it has the ability to infect multiple cell types, and it is now the predominant microsporidian species in *A. mellifera* (Williams et al., 2014). In early spring, many dead and weakened honey bees are found around the hive due to the effects of nosemosis.

During the past few decades, there has been little effort to develop a solution to overcome nosemosis. Until recently, fumagillin, which is derived from the microbe *Aspergillus fumigatus*, was generally used to treat this condition (Whittington & Winston, 2003). Interestingly, in *N. apis*, fumagillin has been shown to work effectively given proper drug administration (Williams et al., 2008), while the results of *N. ceranae* have been more variable. It has become readily apparent that more research needs to be done to find a suitable antibiotic for *N. ceranae* infection of *A. mellifera*.

Thus, the aim of this work was to develop a new drug against *N. ceranaei* under *in vitro* and *in vivo*

environments using different plant extracts of various plants which we listed elsewhere in this manuscript. These plants were selected based on two categories. Category 1, the plants (*Mentha arvensis* L., *Lythrum salicaria* L., *Schisandra chinensis* (Turcz.) Baill., *Perilla frutescens* var. *acuta* Kudo, *Physalis alkekengi* var. *francheti* (Mast.) Hort, *Achillea alpina* (Ledeb)) were selected based on their proven antimicrobial activity (Becker et al., 2005; Helvaci et al., 2010; Johnson et al., 2011; Kim, Kim, & Choi, 2011; Liu, Li, & Hu, 1983; Mocan et al., 2014), whereas the rest of the plants (Category 2) were an arbitrary collection based on their abundant availability and easy accessibility (Silva et al., 2013).

MATERIAL AND METHODS

Extraction

The experimental plants (*Amaranthus mangostanys* L., *Mentha arvensis* L., *Allium senescens* L. var. *senescens*, *Astilboides tabularis* (Hemsl.) Engl., *Veratrum oxysepalum* Turcz., *Achyranthes japonica* (Miq.) Nakai, *Lythrum salicaria* L., *Symphytum officinale* L., *Schisandra chinensis* (Turcz.) Baill., *Perilla frutescens* var. *acuta* Kudo, *Physalis alkekengi* var. *francheti* (Mast.) Hort, *Rheum undulatum* L., *Aster scaber* Thunberg, *Cirsium nipponicum* (Maxim.) Makino, *Achillea alpina* (Ledeb), *Disporum uniflorum* Baker, *Astragalus membranaceus* Bunge var. *membranaceus*, *Aster tataricus* L.f., and *Artemisia dubia* Wall.) were purchased from a Kyungdong oriental herbal market in Seoul, Korea, in December 2012. The identification of the plants was performed using the Wild Vegetable Experiment Station, Gangwon ARES, and a voucher specimen (EDU001–EDU019) was stored at Chungbuk National University, Korea. Dried leaves of each plant (100 g) were treated with 50% ethanol (2 l) three times. We evaporated the solution *in vacuo* to yield 8–13 g of extract. The crude extract was dissolved with dimethylsulfoxid (Sigma-Aldrich, St. Louis, MO, USA).

Reagents and cells

The IPL-LD-65Y cell line (IPL cell) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and was maintained for routine culture in a TC-100 medium (Sigma-Aldrich) with 11% fetal calf serum (FCS, Hyclone Laboratories). The cells were seeded at an initial concentration of 2×10^5 cells/ml in tissue culture flasks (Nunc, Roskilde, Denmark) and incubated at 27°C in a cooling incubator. Cells were routinely passaged every seventh day.

Cell viability test

Various concentrations (12.5 ~ 100 μg/mL) of the plant extracts were used to treat the IPL cells. Measurement of cell viability was done using the Wst-8 based colorimetric assay (Dojindo, Japan), which relies on the ability of living cells to reduce a tetrazolium salt into a soluble, coloured formazan product. The cell suspension with 5×10^4 cells/well was cultured in triplicate in a flat-bottomed 96-well plate for 96 h. The Wst-8 reagent was added to both the cells and the blank samples, which were then incubated at 37°C and 5% CO_2 for 3 h. Next, the level of the dye formed, was measured using a spectrophotometer (Bio-Rad, Hercules, CA) at a wavelength of 450 nm. The blank value without cells was subtracted from each experimental value. Cell viability was expressed by the percentage of live cells compared with that found in the negative controls. The percentage of cell viability was calculated as follows: Cell viability (%) = [OD level of experiment group/OD level of negative control] × 100.

Isolation of *Nosema* spores

Nosema spores were isolated from a naturally infected hive located in the experimental apiary of BEESEN CO., LTD., in Chungnam, Republic of Korea. Isolation of *Nosema* spores from the honey bee midguts was performed as previously described (Gisder et al., 2011). Briefly, after correction of the contents of the midgut, the bees were macerated in distilled water using a tissue grinder, and the suspension was filtered with a 70-μm pore size mesh filter. The large particles in suspension were removed by centrifugation at 12,000 rpm and 1500 rpm, and

then the mixture was re-suspended in distilled water. The number of spores was determined using a hemocytometer chamber.

Identification of *Nosema* spores

A qualitative microscopic diagnosis of spores was performed to detect Nosema-infected bee colonies. To differentiate the spore species, molecular species differentiation was analysed using polymerase chain reaction (PCR), according to the procedure found in previous reports (Genersch et al., 2010; Gisder et al., 2010). Briefly, DNA was isolated using GeneAll Exgene (GeneAll, Seoul, Korea) according to the manufacturer's instructions. The PCR primers used in this study are listed below and were purchased from Bioneer (Daejeon, Korea): sense strand Nosema 5'ggcagttatgggaagtaaca3', anti-sense strand Nosema 5'ggtcgtcacatttcatctct3'; sense strand *N. ceranae* 5' cggataaaagagtc-cgttacc3', anti-sense strand *N. ceranae* 5'tgagcagggttctagggat3'; sense strand *N. apis* 5' ccattgccggataagagagt3', ant-sense strand *N. apis* 5'cacgcattgctgcatcattgac3'. For each PCR, the following sequence was used: preheat 94°C for 2 minutes, 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 45 seconds, with a final extension phase at 72°C for 7 minutes. A variable number of cycles were used to ensure that amplification occurred in the linear phase. The PCR products were separated on a 1.5% agarose gel and visualised by ethidium bromide staining and ultraviolet irradiation.

Infection of IPL cells and application of test substances

Infection of Nosema on IPL cells was performed following the methods of previous reports (Gisder et al., 2010; Williams et al., 2008). Spore germination was induced in all the samples as follows: approximately 1×10^8 *Nosema* spores were suspended in 200 µl of freshly prepared germination buffer (0.5 M sodium chloride, 0.5 M sodium hydrogen carbonate, pH to 6.0 with orthophosphoric acid) (de Graaf et al., 1993) and incubated at 37°C for 15 min to allow spore germination. Centrifugation at 210×g for 5 min was done to harvest the IPL cells. The cell pellet was washed twice with 1 ml of freshly prepared 0.1 M sucrose in 1×phosphate-buffered saline (PBS) and re-suspended in a sucrose buffer at a concentration of 2.5×10^7 cells/ml. The germinating spores (1×10^8) were re-suspended in 100 µl IPL cell suspension (2.5×10^6 cells), and the cell-spore suspension was incubated for 5 min at room temperature. Infected cells were re-suspended in 9.5 ml of a TC-100 cell culture medium supplemented with 11% FCS, 250 µg/ml penicillin/streptomycin, and 250 µ of antibiotic/antimycotic-solution (Sigma Aldrich). Finally, a careful transfer was done of 100 µl of the cell suspension (2.5×10^4 infected cells) into each well of a 96-well microplate. For evaluation, 1 µl of each plant extract was added to the infected cells to achieve the desired final concentrations (50 and 100 µg/mL). Cells were incubated for 72 h at 27°C and, subsequently, their infection status was determined independently both via PCR and microscopic analysis.

Experimental design for the *in vivo* study

At least 300 healthy bees were collected from each of the three different source colonies, carefully transferred into two mesh cages (16.5×16.5×48-inches), and stored at 33 ± 1°C. Microscopic analysis was used to identify any bees that remained uninfected. One group was bulk fed only a 50% sucrose solution, whereas the other group was bulk fed a concentration of 1×10^7 *Nosema* spores mixed in 1 ml of 50% sucrose solution to induce an infection within 48 h. *In vivo* toxicity tests of *A. scaber* Thunberg and *A. dubia* Wall. were performed on uninfected (healthy) bees. After weak anesthetisation with CO_2 to make handling easier, each experimental group was divided into 20 bees. The treatment groups were fed *A. scaber* Thunberg, *A. dubia* Wall., or *A. scaber* Thunberg + *A. dubia* Wall. with 1 ml of 60% sucrose solution for 10 days. To assess their degree of infection, the midguts were introduced into antiseptic microtubes filled with 200 µl distilled water. After thorough grinding, the spores were counted using a hemocytometer and a phase-contrast microscope.

RESULTS

Identification of *Nosema* spores

Nosema spores were isolated from the naturally infected bees. The midgut of all infected bees was macerated in distilled water, and then the *Nosema* spores were isolated by filtration and centrifugation. As shown in Fig. 1, the isolated spores had a similar shape (spindle) as those described in previous reports (Gisder et al., 2010).

Identification of *Nosema* species

To differentiate the spore species, molecular species differentiation was analysed with PCR according to the procedure of a previous report (Gisder et al., 2011; Whittington & Winston, 2003). DNA was extracted from 1×10^4 *Nosema* spores and then amplified with specific primers for *N. ceranae* or *N. apis*, and a universal primer (*Nosema* spp) for *N. ceranae* and *N. apis*. As shown in Fig. 2, DNA from the isolated spores was amplified with a universal Nosema primer and an *N. ceranae* primer. However, DNA was not amplified using an *N. apis* primer. This result indicated that the spores isolated from the midgut of bees was *N. ceranae.*

Cell viabilities of plant extracts on IPL cells

A previous study (Gisder et al., 2010), demonstrated the availability of an IPL cell culture-based screening assay for substances active against *Nosema* species. For such an assay, verification of the substances' toxicity is a prerequisite. Therefore, we performed our initial experiments to demonstrate the cytotoxicity of each extract. Cytotoxicities of plant extracts on IPL cells were measured in a broad concentration range of 12.5–100 μg/ml using Wst-8 assays. Cells were treated with plant extract for 96 h after treatment. The negative control (cell only) group was treated only with dimethyl sulfoxide (DMSO) as a solvent for all extracts; 0.99% DMSO was neither cytotoxic nor active against IPL cells and thus did not influence the analysis of our experiments. As shown in Tab. 1, each extract showed a different cytotoxicity depending on the concentrations. The following showed cytotoxic effects at high concentrations: *A. mangostanys* L., *M. arvensis* L., *A. senescens* L. var. *senescens*, *A. tabularis* (Hemsl.) Engl., *V. oxysepalum* Turcz., *A. japonica* (Miq.) Nakai, *L. salicaria* L., *S. officinale* L., *S. chinensis* (Turcz.) Baill., *P. frutescens* var. *acuta* Kudo, *P. alkekengi* var. *francheti* (Mast.) Hort, and *R. undulatum* L. However, *A. scaber* Thunberg, *C. nipponicum* (Maxim.) Makino, *A. alpina* (Ledeb), *D. uniflorum* Baker, *A. membranaceus* Bunge var. *membranaceus*, *A. tataricus* L.f., and *A. dubia* Wall. did not influence cell viability, even at high concentrations. To rule out an effect of the toxic extract on the IPL cells in live bees, we used extracts of *A. scaber* Thunberg, *C. nipponicum* (Maxim.) Makino, *A. alpina* (Ledeb), *D. uniflorum* Baker, *A. membranaceus* Bunge var. *membranaceus*, *A. tataricus* L.f., and *A. dubia* Wall. for screening of anti-nosemosis activity.

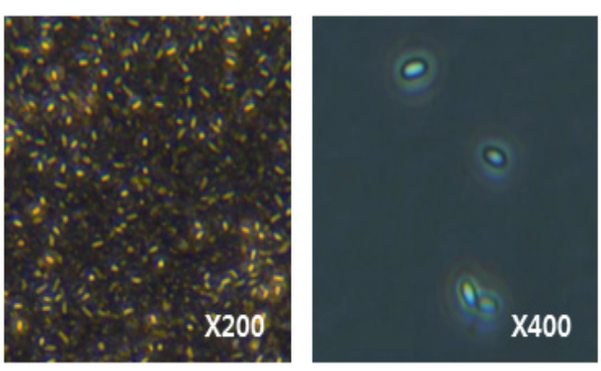

Fig. 1. Microscopic analysis of *Nosema* spores. A *Nosema* spores were isolated from the midgut of honey bees. Representative pictures of *Nosema* spores are shown.

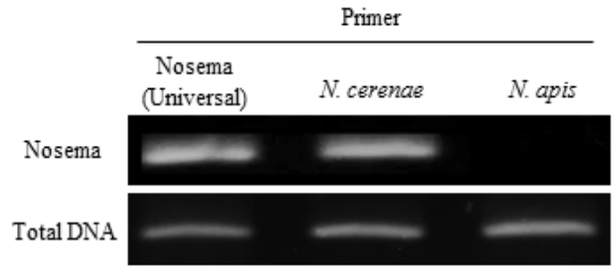

Fig. 2. Results of PCR analysis with a universal primer for *Nosema* species, specific primers for *N. Ceranae,* and specific primers for *N. Apis*. DNA was extracted from 1×10^4 spores, and then the amplified region indicated the specific primer. PCR analyses using a specific primer were performed in triplicate, and all findings showed similar results.

Table 1

The proportion of viable IPL cells (%) after treatments with plant extracts

Name	Concentration (μg/mL)				
	0	12.5	25	50	100
Aster scaber Thunberg	102±12	98±9.2	99±5.3	101±7.3	106±11
Cirsium nipponicum (Maxim.) Makino	98±11	109±11	97±6.6	95±6.3	97±9.3
Amaranthus mangostanys L.	104±9.4	97±2.1	96±9.1	85±6.9	88±12
Achillea alpina (Ledeb)	99±15	96±1.2	101±4.7	102±12	99±7.8
Disporum uniflorum Baker	100±13	108±5.2	102±5.2	99±4.2	96±12
Mentha arvensis L.	102±5.2	91±3.9	87±9.4	90±5.4	85±9.5
Astragalus membranaceus Bunge var. *membranaceus*	105±5.9	101±10	99±3.8	98±7.9	101±7.9
Allium senescens L. var. *senescens*	99±5.2	86±5.4	85±8.5	82±4.3	82±4.5
Astilboides tabularis (Hemsl.) Engl.	100±4.9	90±4.4	82±4.5	79±4.4	75±5.2
Veratrum oxysepalum Turcz.	98±9.3	95±5.3	90±5.3	82±4.8	78±8.9
Achyranthes japonica (Miq.) Nakai	99±3.2	88±4.3	80±5.2	72±5.2	72±5.2
Lythrum salicaria L.	100±9.6	97±4.5	95±4.3	80±4.9	74±9.1
Aster tataricus L.f.	99±8.9	101±6.2	98±4.9	99±9.9	99±5.8
Symphytum officinale L.	100±9.4	83±5.3	82±1.3	76±4.1	70±4.3
Schisandra chinensis (Turcz.) Baill.	99±9.3	97±4.5	88±5.3	85±9.5	83±3.3
Perilla frutescens var. *acuta* Kudo	98±8.2	91±5.4	88±5.3	88±4.3	80±5.2
Physalis alkekengi var. *francheti* (Mast.) Hort	108±5.2	97±4.3	88±5.4	89±9.3	85±4.3
Rheum undulatum L.	104±11	97±4.4	87±12	90±4.7	83±11
Artemisia dubia Wall.	99±2.5	101±4.4	103±12	107±12	99±12

Screening of anti-nosemosis compound in plant extracts

In this study we intended to find an anti-nosemosis drug, which would have few or no cytotoxic effects on live bees. To this end, we performed an IPL cell culture-based screening assay for substances which possess low cytotoxic compounds active against *N. ceranae* (*A. scaber* Thunberg, *C. nipponicum* (Maxim.) Makino, *A. alpina* (Ledeb), *D. uniflorum* Baker, *A. membranaceus* Bunge var. *membranaceus*, *A. tataricus* L.f., and *A. dubia* Wall.). As shown in Tab. 2, the band intensities of the DNA band of *N. ceranae* were decreased by treatment with the *A. scaber* Thunberg or *A. dubia* Wall. extracts. These results indicate that the number of *Nosema* spores was decreased by treatment with *A. scaber* Thunberg or *A. dubia* Wall. However, extracts from *C. nipponicum* (Maxim.) Makino, *A. alpina* (Ledeb), *D. uniflorum* Baker, *A. membranaceus* Bunge var. *membranaceus*, and *A. tataricus* L.f. did not influence the DNA band intensity of *N. ceranae*.

Table 2

Effect of plants extract on the development of *Nosema ceranae*

Name	Band intensity			
	IPL cell only	IPL cell+Spore		
		Concentration (μg/mL)		
		0	50	100
Aster scaber Thunberg	0	42.4	28.3	22.5
Cirsium nipponicum (Maxim.) Makino	0	50.2	49.3	51.1
Achillea alpina (Ledeb)	0	49.7	48.2	46.1
Disporum uniflorum Baker	0	46.1	41.2	45.2
Astragalus membranaceus Bunge var. *membranaceus*	0	51.8	49.2	46.8
Aster tataricus L.f.	0	47.1	44.2	46.2
Artemisia dubia Wall.	0	44.3	39.3	24.2

PCR analyses using a specific primer of *Nosema ceranae* were performed in triplicate, and all of the performances showed similar results. Each PCR band was digitised and expressed as an intensity.

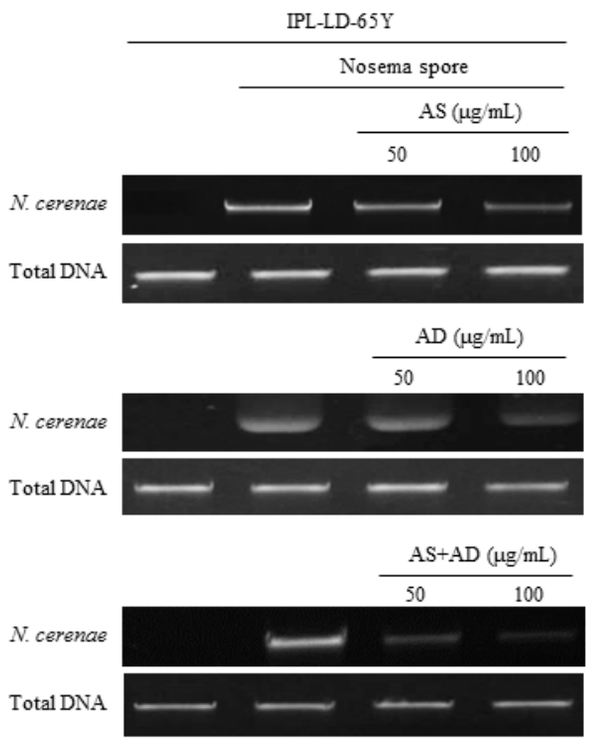

Fig. 3. Effect of *A. scaber* (AS), *A. dubia* (AD), and *A. scaber* + *A. dubia* (AS+AD) on the development of *N. ceranae*. Germinated spores of *N. ceranae* were mixed with IPL-LD-65Y cell line suspension and then treated with plant extracts. PCR analyses using a specific primer were performed in triplicate, and all of them produced similar results.

Fig. 3 shows the representative anti-nosemosis data of *A. scaber* Thunberg (AS) or *A. dubia* Wall. (AD). The thickness of the PCR bands of *N. ceranae* were reduced in a dose dependent manner by AS and AD. We also demonstrated anti-nosemosis activity of the AS and AD mixture. To make this AS and AD mixture, equal amounts of dry plants of AS and AD were combined and then extracted following the description in the Materials and Methods section. Interestingly, the mixture of AS and AD showed stronger activity than either a single substance of AS or AD, for reducing the number of nosema infections.

In vivo activity of A. scaber (AS), and A. dubia (AD)

In advance of this live bee experiment, an *in vivo* toxicity test was performed first. Healthy bees were divided into five groups and then treated with various concentration of AS, AD, or AS+AD mixed in 1 ml of 60% sucrose solution for 10 days. Dimethoate was used as a highly cytotoxic agent. In the untreated colony, only one bee died before the end of the experiment (Fig. 4). However, all bees that received dimethoate died, even at low concentrations. The feeding of AS or AD with a 60% sucrose solution had no influence on the survival rate of bees until a concentration of 25 μg/ml was reached. However, giving 50 and 100 μg/ml of

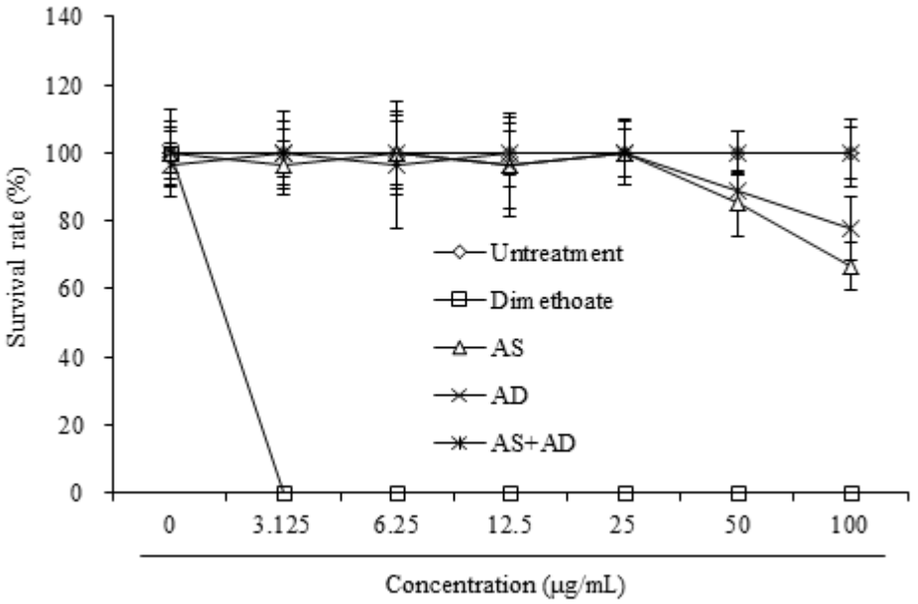

Fig. 4. *In vivo* toxicity of *A. dubia* (AD), and *in vivo* toxicity of *A. scaber* (AS). Healthy bees were divided into five groups and then treated with various concentration of AS, AD, or AS+AD mixed with of 60% sucrose solution for 10 days. The bees' survival rate was calculated as follows: Survival rate (%) = [(number of live bees)/(total number of bees)] x 100. The values that are shown are the means ± SDs of the three independent experiments.

Fig. 5. The influence of *A. dubia* (AD), and the influence of *A. scaber* (AS) on the survival rate of bees infected with *N. ceramae*. In this study, bees that had been infected by *N. ceramae* were treated with AS, AD, or AS+AD in a 60% sucrose solution for 10 days, and then the survival rate of the bees was calculated. The values that are shown are the means ± SDs of the three independent experiments. $*p < 0.05$; $**p < 0.01$ as compared to the untreated bees.

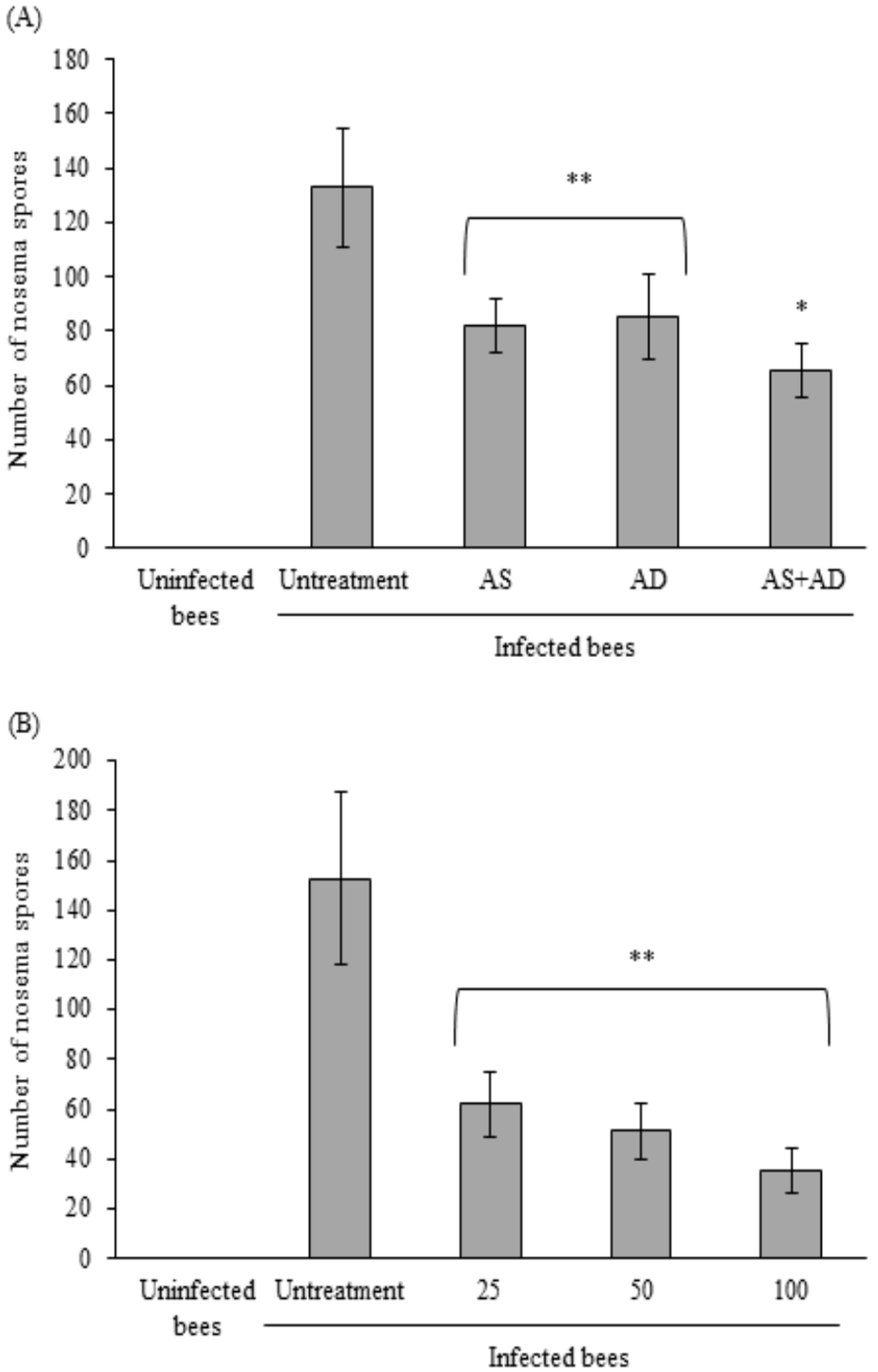

Fig. 6. In vivo activity of *A. dubia* (AD) and *A. scaber* (AS). (A) Infected bees by *N. ceramae* were treated with 25 μg/ml AS, AD, or AS+AD in a 60% sucrose solution for 10 days. The spores, which were derived from the midgut, were counted using a hemocytometer. (B) Infected bees were treated with various concentrations of AS+AD. The values that are shown are the means ± SDs of the three independent experiments. $^{**}p < 0.01$ as compared to the untreated bees.

AS decreased the percentage of live bees to 85% and 67%, respectively. Feeding 50 and 100 μg/ml of AD also decreased the percent of live bees to 89% and 78%, respectively. Interestingly, feeding a mixture of AS + AD did not influence the survival rate of the normal bees at any of the concentrations.

To demonstrate the in vivo anti-nosemosis activity of AS, and AD, we artificially inoculated *Nosema* spores (1×10^7 spores/ml) of AS, and

separately of AD, into 100 bees. All of the uninfected bees survived until the end of the experiment, but only 20% of bees that received no treatment after infection survived. Bees receiving 25 µg/ml of AS, and bees receiving 25 µg/ml of AD had survival rates of 52.5% and 58%, respectively, on Day 10 (Fig. 5). Interestingly, the mixture of AS + AD increased the survival rate of *Nosema* spore-infected bees to 78.1%. *Nosema* spores were observed in all dead bees in the infected groups.

N. ceranae development was monitored as the number of spores present in the midgut of surviving bees at the end of the experiment (Day 10) (Fig. 6). The statistical analyses revealed that the spore content was higher in the infected control group (untreated) than it was in infected bees that were fed 25 µg/ml of AS, AD, or AS+AD. The best inhibition of Nosema proliferation was observed in the AS+AD treatment group (a reduction of ~51%). The honey bees supplemented with a single extract (a reduction of 37% (AS) or 35% (AD)) also presented a significant decrease in their Nosema spore load. As shown in Fig. 6(B), honey bees supplemented with the AS+AD combination presented a significant decrease in the number of spores in a dose-dependent manner. Surprisingly, 100 µg/ml of AS+AD induced a reduction in the parasite load until 77%, without *in vivo* toxicity.

DISCUSSION

In this study, we demonstrated that, depending on the dosage, *A. scaber* extracts as well as *A. dubia* extracts were effective at inhibiting the *in vitro* and *in vivo* development of *N. ceranae*. To the best of our knowledge, our study is the first report on the antimicrosporidian activity of *A. scaber*, and *A. dubia*. Although anti-nosemosis activity of *A. dubia* has not been reported, an ethanol extract of *Artemisia absinthium*, which is included in Artemisia species like *A. dubia*, has been tested against *Nosema* species (Pohorecka, 2004; Porrini et al., 2011). But, there is controversy about the anti-nosemosis activity of *A. absinthium*. Pohorecka (2004) firstly verified anti-nosemosis activity of *A. absinthium* with 5% and 10% extracts on bees both naturally and artificially infected with *N. apis* spores, but *A. absinthium* also had a harmful impact on these insects (Pohorecka, 2004). On the other hand, Porrini et al. (2011) found that an extract of *A. absinthium* did not diminish the spore load of *N. ceranae* infection throughout their experiment, even at the highest concentration (10%). They also reported another new plant (*Laurus nobilis*) for the inhibition of *N. ceranae* development (Porrini et al., 2011). An extract of *L. nobilis* at a 1% concentration inhibited the *in vivo* development of *N. ceranae*. Furthermore, *L. nobilis* extract also showed a low toxicity during the assay. The differences between the two studies (Pohorecka, 2004; Porrini et al., 2011) could be explained by the difference in plant extracts' susceptibility to *Nosema* species (*N. apis* vs *N. ceranae*) or by the extraction method used for the plants (Ahameethunisa & Hopper, 2010; Tariq et al., 2009).

The bioactivity of *A. scaber* and *A. dubia* extracts against other pathogens, such as viruses (Kwon et al., 2000) and bacteria (Satyal et al., 2012), has also been reported. In traditional eastern medicine, *A. scaber* is known to have various medicinal effects. Minced *A. scaber* has been spread on the skin to treat external injuries. Previous studies have demonstrated the effects of an aqueous extract of *A. scaber* on the immune system via enhanced macrophage activation in mice. This extract strangely increased the production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), nitric oxide, and cyclooxigenase-2 (Kim et al., 2009). *A. scaber* extract also showed anti-oxidant activities depending on the manufacturing process (Kim, Choi, & Park, 2014). Even an anti-HIV-1 effect was revealed for treatment using *A. scaber* derived caffeoyl quinic acid (Kwon et al., 2000).

In addition to the herbs, a few chemicals have been reported as anti-nosemosis therapeutic compounds. Representative examples may include oxalic acid and acetic acid. Nanetti et al. (2015) tested the efficacy of an oxalic acid solution against artificial and natural infections. In an *in vitro* study, the administration of oxalic acid hindered the development of *N. ceranae*

infections compared to the untreated controls, which confirmed the results obtained in the field. When oxalic acid was administered to free flying colonies twice, the infection prevalence decreased in both young and old bees. At the same time, an increased prevalence in all the untreated controls was detected. Interestingly, the bees treated with oxalic acid overwintered healthily, but the untreated ones did not. Traditionally, acetic acid has been used to disinfect honeycombs to remove *Nosema* spores, but this has not been verified in scientific experiments.

Fumagillin, an antibiotic isolated from the fungus *A. fumigatus*, has been the only widely used method to treat colonies infected with *Nosema* species in western honey bees, *A. mellifera* (Bailey, 1953; Higes et al., 2011). It is considered to be the most effective medication for *N. apis* infection in honey bees (Bailey, 1953). However, in contrast to fumagillin's efficacy against *N. apis* in honey bees, *N. ceranae* might evade fumagillin (Huang et al., 2013; Williams et al., 2008) therefore, this drug may be inappropriate for the treatment of *N. ceranae* infections. It may be inappropriate, even though previous reports have shown that fumagillin treatment of colonies infected with *N. ceranae* was effective (Botías et al., 2013; Higes et al., 2008). It has even been considered that fumagillin can contribute to a higher incidence rate and pathogenicity of *N. ceranae* (Huang et al., 2013). For these reasons, most researchers want to find a new agent to use against *N. ceranae*.

Several antimicrobial agents (albendazole, ornidazole, tinidazole, and metronidazole) were tested to determine their activity against *N. ceranae* in *Nosema* spore-infected cells and therefore to identify new drugs with anti-nosemosis activity that is comparable to that of fumagillin. The antimicrobial agents were selected based on their well-known inhibitory effects on other fungi and microsporidia. Albendazole had no significant *in vitro* activity against *N. ceranae*, and ornidazole also had no significant *in vitro* activity against *N. ceranae*. At a concentration of 2 mg/ml, tinidazole or metronidazole can completely inhibit *N. ceranae* infection of IPL cells similar to the activity of fumagillin *in vitro*. However, both substances are unlikely to have a future as anti-nosemosis drugs in honey bees because many countries prohibit the use of nitroimidazoles in domestic animals (Payne et al., 1999).

In this study, we identified two anti-nosemosis plants, *A. scaber*, and *A. dubia*, which when separately used had reduced the spore development of *N. ceranae* in spore-infected cells. The most important finding was that these plants were effective at non-toxic concentrations. Anti-nosemosis activities of both plants were revealed in a honey bee experiment. Specifically, the mixture containing both *A. scaber* + *A. dubia* showed stronger activity than treatment with only a single plant. However, the mechanisms of action of *A. scaber*, and the mechanisms of action of *A. dubia* against *N. ceranae* are still unclear. Further research will be necessary to determine the identity of these active compounds. Our results suggest new possibilities for medicaments and therapeutic methods to control *N. ceranae* infection in honey bees.

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