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Original Article

ANTI-NOSEMOSIS ACTIVITY OF *ASTER SCABER* AND *ARTEMISIA DUBIA* AQUEOUS EXTRACTS

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Abstract

In our previous study, we demonstrated that the ethanol extracts of *Artemisia dubia* (*A. dubia*) and *Aster scaber* (*A. scaber*) have anti-nosemosis activity. In our present study, we intend to establish the anti-nosemosis activity of aqueous, ethyl acetate (EA), and butanol (BuOH) extracts of *A. dubia* and *A. scaber*. In order to determine the optimal dose, we performed both *in vitro* and *in vivo* toxicity for all the extracts and also carried out anti-nosemosis experiments. Although all of the extracts (aqueous, EA, and BuOH) showed *in vitro* and *in vivo* anti-nosemosis activity in a dose-dependent manner, the aqueous extracts of *A. dubia* and *A. scaber* showed more potent anti-nosemosis activity than the EA and BuOH extracts. Moreover, an aqueous extract of *A. dubia* + *A. scaber* demonstrated stronger anti-nosemosis activity compared with the aqueous extracts of either *A. dubia* or *A. scaber* alone. Although the main ingredients in *A. dubia* and *A. scaber* remain unclear, our results suggest that the active components of *A. dubia* and *A. scaber* could dissolve in the aqueous fraction.

Keywords: *Artemisia dubia*, *Aster scaber*, *Nosema ceranae*, nosemosis

INTRODUCTION

Nosemosis is a disease of adult bees caused by *Nosema* species which belongs to the class of Microsporidia of the fungal kingdom (Sprague & Becnel, 1998; Sprague & Becnel, 1999). The genus *Nosema* contains 322 species including the sub-species prevalent in honey bees *Nosema ceranae* (*N. ceranae)* and *Nosema apis* (*N. apis)* (http://www.indexfungorum.org). *N. apis* is a parasite of the western honey bee (*Apis mellifera*) and *N. ceranae* is a parasite of the eastern honey bee (*Apis ceranae*). Honey bees are important pollinators and crucial to the food supply (Calderone, 2012), and those affected by *Nosema* species can be found across the world. Generally, a spore of *N. apis* reproduces in the midgut epithelium of the honey bee. However, this is not the only anatomical location where it can survive, as; it also appears in fat body, the alimentary canal, malpighian tubules, hypopharyngeal glands and salivary glands; (Chen & Huang, 2010; Ptaszyńska et al., 2012). *N. ceranae* is considered a more dangerous microsporidian than *N. apis* due to its potential to infect the entire body of *A. mellifera* (Williams et al., 2014). Nosemosis is most common during the spring and autumn than summer, which could be due to the excess energy consumed by the bees to enhance the immune defense for combatting any microbial attack. Additionally, higher humidity and colder temperatures during spring was also one of the reasons that decreased the rate of nosemosis in summer (Ptaszyńska, Paleolog, & Borsuk, 2016). During these seasons, brown feces due to dysentery, a common sign of nosemosis, were found in the comb and around the hive (Klee et al., 2007).

A beekeeper can recognize the nosemosis infection when there are weakened and dead bees found around the hive, and then the prognosis at this point is severe.

There have been a few attempts to develop nosemosis therapy. The traditional approach is to remove the *Nosema* infection through the sterilization of the hives (with boiling water, 6% soda, and blue flame) and destruction of the combs after the infected bees are killed. Since the discovery of its anti-nosemosis effects, fumagillin has been considered the first treatment choice (Whittington & Winston, 2003). Williams et al. (2008) reported that the appropriate administration of fumagillin efficiently combats *N. apis*, but its activity against *N. ceranae* is not very promising (Williams et al., 2008). Thus, there is a need to find a suitable substance to combat *N. ceranae* infection of *A. mellifera*. Anti-nosemosis effects has been showed for caffeine (Strachecka et al., 2014a), curcumin (Strachecka, Olszewski, & Paleolog, 2015), coenzyme Q10 (Strachecka et al., 2014b) and also ethanol extracts of *Aster scaber* (*A. scaber*) and *Artemisia dubia* (*A. dubia*) (Kim et al., 2016).

In our previous study (Kim et al., 2016) we had reported the anti-nosemosis activity of *A. scaber* and *A. dubia* in ethanol solvent, since it is a polar solvent whose extract consists of both polar and non-polar compounds. Thus in our current study, we sequentially extracted the *A. scaber* and *A. dubia*, based on the polarity of solvents (water, butanol (BuOH) and ethyl acetate (EA)) and they were screened for *in vitro* and *in vivo* anti-nosemosis activity. IPL cell culture is a perfect tool to screen the activity of substances against *Nosema* species, so we employed these cells for *in vitro* anti-nosemosis analysis (Gisder et al., 2010; Kim et al., 2016).

MATERIAL AND METHODS

Plant material

A. scaber and *A. dubia* were purchased from the Kyungdong Oriental Herbal Market in Seoul, Korea in April 2016. The plants were identified at the Wild Vegetable Experiment Station, Gangwon ARES, and the voucher specimen was deposited at Chungbuk National University, Korea.

Plant extraction preparation

Whole plants of *A. dubia* and *A. scaber* were shade-dried for one week and powdered by using a blender. To obtain aqeous extract, about 20 g of *A. dubia* and *A. scaber* were soaked in 400 mL of water and reflux extraction was carried out at 100°C for three hours. The concentrated aqueous extracts of both plants were lyophilized separately to obtain final powdered form. The lyophilized plant powders were soaked in water and EA at 1:1 ratio in a separating funnel, where the EA portion was separated and evaporated *in vacuo* to yield 8-13 g of extract. The same procedure was followed to obtain BuOH extract, where EA was replaced with BuOH, and the final yield of the BuOH extract was 8-13 g. These extracts were then dissolved in dimethylsulfoxid (Sigma-Aldrich, St. Louis, MO, USA) for further estimation.

Reagents and maintenance of cells

The IPL-LD-65Y cell line (IPL cell) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and 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. The cell pass was carried out on every seventh day.

Cell viability test

The cell viability test was conducted by using the IPL-LD-65Y cell line, where the cell line was treated with various concentrations (0.625-10 µg/mL) of the plant extracts. The cell viability was measured using the Wst-8 based colorimetric assay (Dojindo, Japan), which was based on the ability of live cells to reduce tetrazolium salt into a soluble colored formazan product. The cell suspension with 5×10^4 cells/well was cultured in triplicate in a flat-bottomed 96-well plate for 96 hours. The Wst-8 reagent was added to both the cells and the blank samples, which were then incubated for three hours at 37°C and 5% CO_2.

The level of the dye formed was the measured using a spectrophotometer (Bio-Rad, Hercules, CA) at a wavelength of 450 nm. The blank values without cells were 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 experimental 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. *Nosema* spores were isolated from the honey bee midguts as previously described (Gisder et al., 2011). Briefly, after dissection the midgut contents were collected, and macerated in phosphate-buffered saline (PBS) with the use of a tissue grinder, and the suspension was filtered with a 70-μm mesh filter. Then, the suspension was centrifuged at a range of 1500 to 12000 rpm to remove large particles, and the mixture was resuspended in distilled water to calibrate the number of spores using a hemocytometer.

Identification of *Nosema* spores

A qualitative microscopic diagnosis of the spores was performed to detect *Nosema*-infected bee colonies. To differentiate the spore species, molecular species differentiation was analyzed with the use of a polymerase chain reaction (PCR); according to the procedure of previous reports (Genersch et al., 2010; Gisder et al., 2010). The DNA was isolated using GeneAll Exgene (GeneAll, Seoul, Korea) per the manufacturer's instructions. Briefly, 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* species) for *N. ceranae* and *N. apis*. The PCR primers used in this study are listed below and were purchased from Bioneer (Daejeon, Korea):

sense strand *Nosema* 5'-GGCAGTTATGGGAAGTAACA-3',

anti-sense strand *Nosema* 5'-GGTCGTCACATTTCATCTCT-3';

sense strand *N. ceranae* 5'- CGGATAAAAGAGTCCGTTACC-3',

anti-sense strand *N. ceranae* 5'-TGAGCAGGGTTCTAGGGAT-3';

sense strand *N. apis* 5'- CCATTGCCGGATAAGAGAGT-3',

ant-sense strand *N. apis* 5'-CACGCATTGCTGCATCATTGAC-3'.

Each PCR was preheated to 94°C for 2 minutes followed by 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 visualized by ethidium bromide staining and ultraviolet irradiation.

Infection of IPL cells and application of test extracts

Previously described methods were followed to infect the IPL cells with *Nosema* (Gisder et al., 2010; Williams et al., 2008). To induce spore germination in every sample 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) followed by 15-minute incubation at 37°C to allow spore germination (de Graaf et al., 1993). After incubation, the IPL cells were harvested by centrifugation at 210×g for five minutes. The cell pellet was then washed twice with 1 mL of freshly prepared 0.1 M sucrose in 1×PBS buffer and resuspended in a sucrose buffer at a concentration of 2.5×10^7 cells/mL. The germinating spores (1×10^8) were resuspended in 100 μL of the IPL cell suspension (2.5×10^6 cells), and the cell-spore suspension was incubated for five minutes at room temperature. Infected cells were resuspended in 9.5 mL of a TC-100 cell culture medium supplemented with 11% FCS, 250 μg/mL penicillin/streptomycin, and 250 μL antibiotic/antimycotic-solution (Sigma Aldrich). Finally, 100 μL of the cell suspension (2.5×10^4 infected cells) was carefully transferred into each well of a 96-well microplate. For evaluation, 1 μL of EA and BuOH extract of *A. dubia* and *A. scaber* were added to the mixture of germinating spores and IPL cell suspension

in a TC-100 medium to achieve the desired final concentrations (0.625-10 µg/mL). The cells were then incubated for 72 hours at 27°C, and their infection status was subsequently determined via microscopic and PCR analysis.

To perform microscopic analysis, infected cells and floating spores were harvested from each well and the number of spores ware counted using a hemocytometer. PCR analysis was performed after the isolation of DNA from harvested infected cells and floating spores. The percentage of inhibition by microscopic analysis was calculated as follows: Inhibition rate (%) = [((number of *Nosema* spores in the treated cells at the initial stage - number of *Nosema* spores in the treated cells after 72 hours) / (number of *Nosema* spores in the untreated cells at initial stage - number of *Nosema* spores in the untreated cells after 72 h) × 100)-100] × -1.

Experimental design of the *in vivo* study

At least 300 healthy bees were collected from each of the three different source colonies and they were carefully transferred to two mesh cages (16.5×16.5×48 inches), and stored at 33 ± 1°C. Microscopic analysis was carried out to identify the normal bees and included in our experiment.

Prior to the anti-nosemosis experiment with live bees, an *in vivo* toxicity test was performed in which about twenty honey bees per cage were employed and usually treated in the morning by 10 am. While normal control was fed with 50% sugar solution alone; cage 2 was fed with fumagilin (fumidil B) (20 mg/mL), a commercial reference drug. The rest of the cages were treated with *A. dubia* water extract 0.125 - 10 (µg/mL); *A. scaber* water extract 0.125 - 10 (µg/mL); *A. dubia* BuOH extract 0.125 - 10 (µg/mL); *A. scaber* BuOH extract 0.125 - 10 (µg/mL); *A. dubia* EA extract 0.125 - 10 (µg/mL); *A. scaber* extract 0.125 - 10 (µg/mL). The live bees were counted at 0, 24, 48, 72, 96 and 120 hrs. The results were expressed in percentage.

In vivo activity tests of *A. scaber* and *A. dubia* were performed with uninfected (ie, healthy) bees. The study lasted one week (2 day for *Nosema* infection induction and 5 days for treatment) and on day 0 the bees were split into two experimental groups each of twenty bees. On the same day, group 1 was fed with only 50% sucrose solution and considered normal control and group 2 was fed with *Nosema* spores $1x10^7$ in 50% sucrose solution for 48 hrs to induce nosemosis. Afterwards, cage 1 was considered normal control and fed with DMSO dissolved in 50% sucrose solution only, and cage 2 was *Nosema* infection control. The rest of the cages of the *Nosema* infected honey bees were treated with *A. dubia* water extract (0.125, 0.250, 0.50, 1 µg/mL); *A. dubia* BuOH extract (0.125, 0.250, 0.50, 1 µg/mL); *A. dubia* EA extract (0.125, 0.250, 0.50, 1 µg/mL) and *A. scaber* water extract (0.125, 0.250, 0.50, 1 µg/mL); *A. scaber* BuOH extract (0.125, 0.250, 0.50, 1 µg/mL); *A. scaber* EA extract (0.125, 0.250, 0.50, 1 µg/mL) and fumagillin (20mg/mL). The treatment usually occurred in the morning by 10 am. After one weak of the experiment, all the normal infection control and treated honey-bee cages were anesthetized with CO_2 to facilitate handling. To assess the anti-nosemosis activity, the three honey-bee midguts were dissected and introduced into antiseptic micro tubes filled with 200 µL distilled water. After thorough grinding, the spores were counted using a hemocytometer under a phase-contrast microscope.

RESULTS

Identification of *Nosema* species

The spindled shaped spores isolated from naturally infected bees were nosema (Fig 1A). To differentiate the spore species, molecular species differentiation was performed with PCR following the procedure of previous reports (Gisder et al., 2011; Whittington & Winston, 2003). As shown in Fig. 1B, DNA from the isolated spores was amplified with a universal *Nosema* primer and *N. ceranae* primer. However, DNA was not amplified using an *N. apis* primer.

Viability of IPL cells with plant extracts

Since toxicity studies for this assay were paramount, we evaluated the cytotoxicity of all the extracts in a broad range of concentrations (0.625-10 µg/mL) using Wst-8 assays. Concentrations below 1% DMSO did not influence the

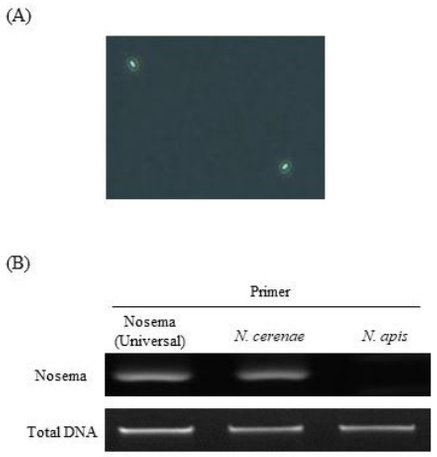

Fig. 1. Identification of *Nosema* species. A *Nosema* spore was isolated from the midgut of the honey bees and confirmed by microscope (A). Representative pictures of *Nosema* spores are shown. DNA was extracted from 1×10^4 spores, and then the amplified region indicated the specific primer. PCR analyses using a specific primer were performed in triplicate, and all findings showed similar results (B).

viability of the IPL cells. As shown in Fig. 2, each extract of *A. dubia* (A) and *A. scaber* (B) showed a concentration-dependent cytotoxicity, but there was no effect on cell viability, even at high concentrations.

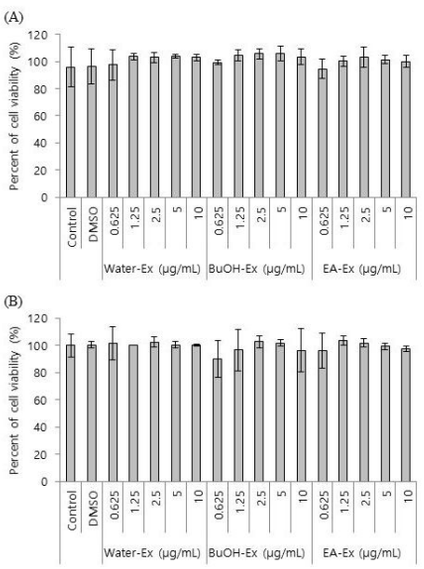

Fig. 2. *In vitro* toxicity of water, BuOH and EA extracts of *A. dubia* (A) and *A. scaber* (B). The IPL cells were treated with various concentrations of the plant extracts, and cell viability was measured using the Wst-8 based colorimetric. The values that are shown are the means ± SDs of the three independent experiments.

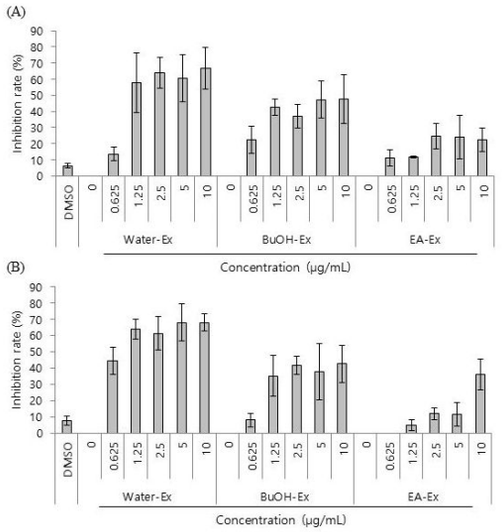

Fig. 3. Effect of water, BuOH and EA extracts of *A. dubia* (A) and *A. scaber* (B) on the development of *Nosema* spore. Germinated spores of *N. ceranae* were mixed with IPL cell line suspension and then treated with plant extracts. Inhibition rate of spore population was determined by microscopic analysis with hemocytometer. The values that are shown are the means ± SDs of the three independent experiments.

***In vitro* screening of anti-nosemosis extracts**

Fig. 3 exhibits the *in vitro* anti-nosemosis activity of the extracts of *A. dubia and A. scaber*. Although the level of activity varied, all of the extracts of *A. dubia* (Fig. 3A) and *A. scaber* (Fig. 3B) showed anti-nosemosis activity in a dose-dependent manner. Interestingly, the anti-nosemosis activity of both plants increased in the order of aqueous, BuOH and EA. The aqueous extract of *A. dubia* and *A. scaber* reduced the population of *N. ceranae* to 33-34%, the BuOH extract to 53-58%, and the EA extract to 64-76% of the original population. There was no significant difference in the anti-nosemosis activity of *A. dubia* and *A. scaber* at the highest concentration.

To confirm the anti-nosemosis activity of *A. dubia* and *A. scaber*, PCR analysis was performed in the highest concentration of each extract. As shown in Fig. 4, the band intensities of the *N. ceranae* DNA decreased after treatment with aqueous extracts of *A. dubia* (Fig. 4A) or *A. scaber* (Fig. 4B). However, the extracts of BuOH and EA did not significantly influence the DNA band intensity of *N. ceranae*.

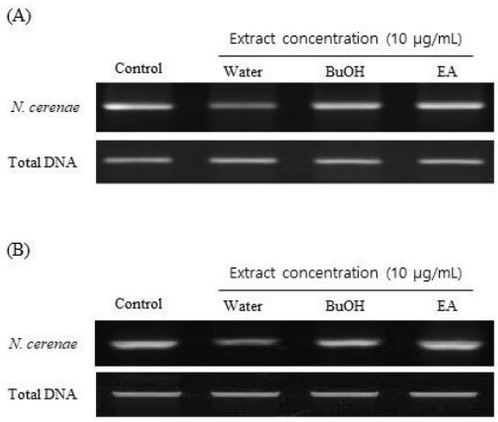

Fig. 4. Anti-nosemosis screening by PCR analysis. In the same condition with Fig. 3, PCR analyses using a specific primer were performed in triplicate, and all of them produced similar results.

In vivo toxicity of each extract of *A. dubia* and *A. scaber*

In the untreated control group, more than 90% of the bees survived during the experimental period (Fig. 5). At the same time, the Fumagillin-treated group showed intermediate toxicity after 120 hours of incubation. Interestingly, all extracts up to a concentration of 1 μg/mL in 50% sucrose solution showed the survival rate of bees between 81.3-98.1%. However, increasing the concentrations of extracts to 2-10 μg/mL caused a slight decrease in the percentage of live bees to 63-79% of the original population. In particular, feeding 10 μg/mL of the *A. dubia* BuOH extract showed the lowest percent of live bees (63%). Concentrations from 0.125-10 μg/mL of each of the extracts showed the following live bee viability: aqueous extract of *A. dubia* 75-90%, BuOH extract of *A. dubia* 63-98%, EA extract of *A. dubia* 68-89%, aqueous extract of *A. scaber* 68-88%, BuOH extract of *A. scaber* 64-95%, and EA extract of *A. scaber* 65-86%. The aqueous extract showed lower toxicity than either the BuOH or EA extracts.

In vivo activity of three different kinds of extract

Fig. 6 shows the anti-nosemosis activity of different extracts of *A. dubia and A. scaber,* where the DMSO-only control group had the highest number of *Nosema* spores compared with the groups treated with extracts. Among the three kinds of solvents used for extraction

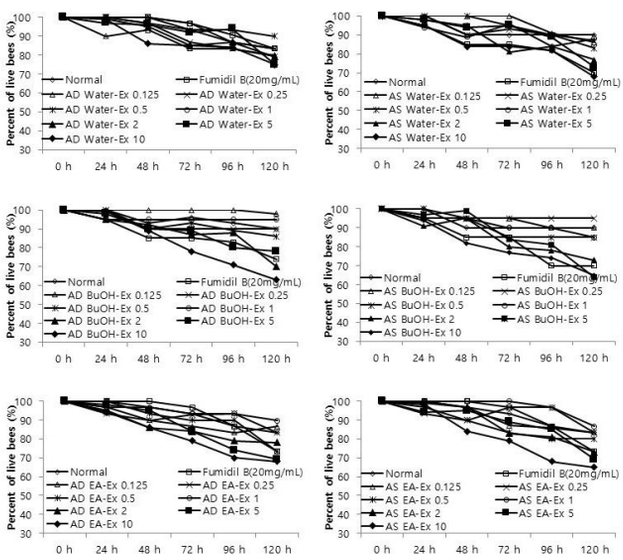

Fig. 5. *In vivo* toxicity of each extracts. Healthy bees were divided into nine groups and then treated with various concentrations of each extract (*A. dubia* : AD, *A. scaber* : AS) with 50% sucrose solution for 5 days. The survival rate was calculated on the fifth day as follows: Survival rate (%) = [(number of live bees)/(total number of bees)] x 100. The values that are shown are the means ± SDs of the three independent experiments.

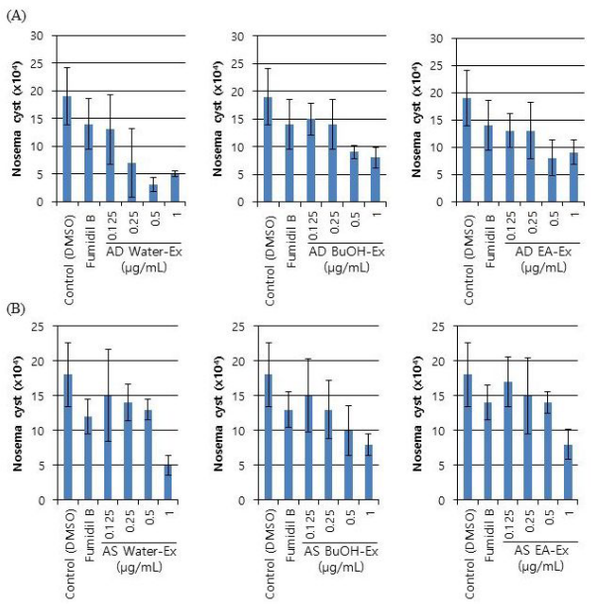

Fig. 6. *In vivo* activity of each extract. Infected bees by *N. ceramae* were treated with 0.125 - 1 μg/ml extracts of *A. dubia* (A) and *A. scaber* (B) in a 50% sucrose solution for 5 days. The spores, which were derived from the midgut, were counted using a hemocytometer. The values that are shown are the means ± SDs of the three independent experiments. **p < 0.01 as compared to the untreated bees.

(aqueous, BuOH, and EA), the highest inhibition of spore proliferation was observed in the groups treated with aqueous extracts. Notably, 1 μg/mL of the aqueous extract of *A. dubia* reduced the number of spores by around 75%. The aqueous extract of *A. scaber* also reduced the number of spore by almost 72% at the 1 μg/mL concentration. The positive control drug, fumagillin (20 mg/mL), showed less anti-nosemosis activity (27-33%), whereas the BuOH and EA extracts of *A. dubia* reduced the number of spores by 57% and 52%, respectively. The BuOH and EA extracts of *A. scaber* reduced the number of spores by 55% and 54%, respectively.

In vivo* activity of aqueous extract of *A. dubia* + *A. scaber

In order to evaluate the anti-nosemosis activity of the aqueous extracts, the aqueous extract of *A. scaber* + *A. dubia* was prepared in addition to the *A. scaber* and *A. dubia* extracts. As shown in Fig. 7, 85% of the untreated, uninfected control bees survived for 120 hours, but only 70% of the fumagillin-treated bees were survived. Interestingly, none of the three extracts tested showed toxicity at any concentration. Even in bees treated with *A. scaber* 1 μg/mL, showed a less toxicity with 85% survival rate.

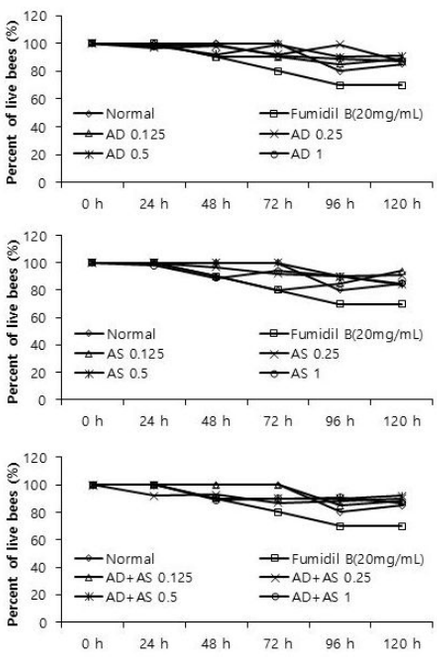

Fig. 7. *In vivo* toxicity of water extracts. Healthy bees were treated with water extracts of *A. dubia* (AD), *A. scaber* (AS) and *A. dubia* + *A. scaber* (AD+AS) as in Fig. 5. The survival rate was represented by percent of live bees. The values that are shown are the means ± SDs of the three independent experiments.

The anti-nosemosis activity of each extract was analyzed using the same methods depicted in Fig. 6. Fumagillin (20 mg/mL) was used as a positive control drug. As shown in Fig. 8, all of the aqueous extracts exhibited anti-nosemosis activity. Among the three aqueous extracts, those treated with both *A. scaber* + *A. dubia* showed the highest inhibition of spore proliferation. The aqueous extracts of *A. dubia* and *A. scaber* alone also reduced the number of spores by almost 58% and 64%, respectively at 1 μg/mL. However, the aqueous extract of *A. dubia* + *A. scaber* reduced the number of spores by almost 76% at 1 μg/mL.

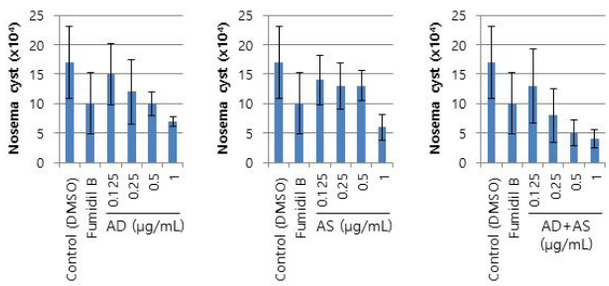

Fig. 8. *In vivo* activity of water extracts. Infected bees by *N. ceramae* were treated with water extracts of *A. dubia* (AD), *A. scaber* (AS) and *A. dubia* + *A. scaber* (AD+AS) in a 50% sucrose solution for 5 days. The spores, which were derived from the midgut, were counted using a hemocytometer. The values that are shown are the means ± SDs of the three independent experiments. **p < 0.01 as compared to the untreated bees.

DISCUSSION

In our previous paper (Kim et al., 2016), we prepared ethanol extracts of *A. scaber*, *A. dubia* and *A. scaber* + *A. dubia,* and demonstrated the anti-nosemosis activity of both *A. scaber* and *A. dubia* at non-toxic concentrations. Although both *A. scaber* and *A. dubia* separately had showed anti-nosemosis activity, combined *A. scaber* + *A. dubia* exhibited even stronger activity. Therefore in the present study, we

have tried to demonstrate the chemical characteristics of the active compounds in each extract. In our previous study, 100 µg/mL of the ethanol extract of *A. scaber* + *A. dubia* showed 77% spore reduction without *in vivo* toxicity, but as expected, the aqueous extract of *A. dubia* + *A. scaber*, in which BuOH and EA soluble compounds were eliminated, reduced the number of spores to almost 76% at 1 µg/mL. As a result, the active molecule had to dissolve in water. Both the BuOH and EA extracts also showed anti-nosemosis activity albeit not as strong as the aqueous extracts. These results indicate that *A. dubia* and *A. scaber* have more than one anti-nosemosis compound with different solubility in each solvent (aqueous, BuOH, and EA).

The anti-nosemosis effects of *A. dubia* and *A. scaber* have not been reported except in our previous paper. As *A. dubia, Artemisia absinthium* has also been tested against *Nosema* species by two different research groups (Pohorecka, 2004; Porrini et al., 2011). Pohorecka (2004) first reported that an ethanol extract of *Artemisia absinthium* inhibited *N. apis*, and Porrini et al., (2011) reported the anti-nosemosis activity of *Artemisia absinthium* seven years later.

Unfortunately, the results of these two research teams are inconsistent. While Pohorecka (2004) reported that *Artemisia absinthium* had an antimicrobial effect, Porrini et al. (2011) reported no such effect. Porrini and his colleagues (2011) reported that different susceptibilities of *N. ceranae* and *N. apis* to the ethanol extract of this herb or other factors that could influence the chemical composition (eg, the extraction method) might explain this difference between the two studies Ahameethunisa & Hopper, (2010) and by Tariq et al., (2009). But in our opinion, *A. scaber* which contains five important secondary metabolites namely, Caffeoyl quinic acid, (-) 3, 5-dicaffeoyl-muco-quinic acid, (-) 3, 5-dicaffeoyl quinic acid, (-) 4, 5-dicaffeoyl quinic acid, (-) 5-caffeoyl quinic acid with anti-fungal property, and *A. dubia* composed of caffeic acid, gallic acid, catechin, coumarin, and camphor could act against fungus. *Nosema*'s place in the fungi kingdom lead us to speculate that both *A. scaber* and *A. dubia* could have anti nosemosis activity (Kwon.et al., 2000; Rhimi et al., 2017; Sardi et al., 2016; Li et al., 2017; Hirasawa & Takada, 2004; Montagner et al., 2008; Mahilrajan et al., 2014; Kiani et al., 2016).

Fumagillin, isolated from the microbial organism *A. fumigatus,* has been used against a variety of microsporidial parasites in both bee and human medicine. It has also been shown to inhibit angiogenesis (Chung et al., 1993) and thus been studied in cancer treatment research. Moreover, it has been the most commonly used medicinal product in the treatment of *Nosema* infection in western honey bees, *A. mellifera* (Bailey, 1953; Higes et al., 2011).

Although the most valuable compound in human medicine and apiculture, fumagillin is not free from such side effects as gastrointestinal cramping, diarrhea, and significant weight loss, and has limited application in humans (Chung et al., 1993; Molina et al., 2000; Molina et al., 2002; Yanase et al., 1993). Moreover, chromosomal aberrations and genotoxic potential have been observed in mice (Kulić et al., 2009; Stanimirović, 2010). Enhanced *Nosema* species infection leads to increased fumagillin sales, and residues of fumagillin have been detected in harvested apicultural products (Lopez et al., 2008). Therefore, the potential fumagillin contamination of apicultural products intended for human consumption could be eliminated by the development of alternative treatments against *Nosema* species.

Fumagillin is used as a primary treatment for *Nosema* infection, but the latest report has shown it to be ineffective against *N. ceranae* (Huang et al., 2013). Some reports state that *N. ceranae* can regrow six months after a treatment is terminated, despite some evidence that *N. apis* has never developed a resistance to fumagillin (Higes et al., 2011; Pajuelo, Torres, & Bermejo, 2008). Neither susceptibility nor quick recuperation after fumagillin treatment could account for the replacement of *N. apis* by *N. ceranae*, which has seemingly occurred in North America and elsewhere (Chen et al., 2009; Huang et al., 2008; Klee et al., 2007).

Therefore, in this study, we suggested two anti-

nosemosis plants, *A. scaber* and *A. dubia*, which reduced the spore development of *N. ceranae* in both *in vitro* and *in vivo* experiments. In particular, the mixture of *A. scaber* and *A. dubia* showed stronger activity than treatment with only a single plant, which could be due to the synergetic activity of secondary metabolites present in *A. scaber* and *A. dubia*. Moreover, the anti-nosemosis effects are confirmed to be better when the extracts are dissolved in water compared with butanol or ethyl acetate. The mechanisms of action of *A. scaber* and *A. dubia* against *N. ceranae* remains unclear, and further research is necessary to identify these active compounds. In conclusion, our results suggest new possibilities for controlling *N. ceranae* infection in honey bees.

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