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Effect of *Bidens pilosa* on infection and drug resistance of *Eimeria* in chickens

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ABSTRACT

Extensive use of current anti-coccidial drugs together with drug resistance and residue has raised concerns about public health and poultry development. Here, we studied the anti-coccidial properties of *Bidens pilosa*. A phytochemical approach was developed for analysis of *B. pilosa* utilized as a feed additive. The protective effects of *B. pilosa* supplemented chicken diet were evaluated chickens infected with *Eimeria tenella*. *B. pilosa*, at doses of 0.5%, 1% and 5% of the chicken diet, significantly protected against *E. tenella* as measured by reduction in mortality, weight loss, fecal oocyst excretion and gut pathology in chickens. Finally, drug resistance of *E. tenella* to *B. pilosa* was assessed in chickens using the anti-coccidial index. This index showed that *B. pilosa* induced little, if any, drug resistance to *Eimeria* in chickens. Collectively, this work suggests that *B. pilosa* may serve as a novel, natural remedy for coccidiosis with low drug resistance in chickens.

1. Introduction

Coccidiosis is a disease that has a large economic impact on the poultry industry causing high mortality, poor growth and high medical costs (Williams, 1998). In chickens, coccidiosis is caused by parasites of the genus *Eimeria* (Coccidia subclass). Currently, the use of anti-coccidial drugs is one common means to prevent and treat coccidiosis. However, massive and long-time use of anti-coccidial drugs has led to the presence of drug-resistant parasites and residual drugs in chicken products, raising concerns about public health and food safety (Chapman, 1997; McDonald and Shirley, 2009; Orengo et al., 2012). In European countries, the use of anti-coccidial and anti-histomonas drugs as feed additives has been strictly limited since 2006 (Regulation 1831/2003 of the European Parliament) and a full ban has been proposed to be effective in 2021 by the Council Directive of 2011/50/EU published in the Official Journal of the European Union, L 104 of 19 April 2011. The utilization of anti-coccidial vaccines is an alternative means to prevent coccidiosis. Despite the significant progress made over recent years, efficacy, safety and cost effectiveness are still challenges for anti-coccidial vaccines in poultry (Sharman et al., 2010).

Given the concern voiced by consumers and poultry farmers about the use of the present anti-coccidial agents, there is an urgent need for novel and alternative approaches to prevent and treat coccidiosis in fowl. Reports have indicated that the use of effective, edible herbs and natural products as coccidicides in poultry production can be easily appreciated and accepted by consumers (Hassan et al., 2008; Orengo et al., 2012). Plants have been an extraordinary source of food and medicines for humans and animals since antiquity. Over the past decade, over 20 herbs have been tested for anti-coccidial activities (Akhtar et al., 2012; Allen, 2003; Allen et al., 1997; del Cacho et al., 2010; Lee et al., 2011; Naidoo et al., 2008; Orengo et al., 2012; Remmal et al., 2011; Youn and Noh, 2001). Although some plants showed high toxicity or little or no anti-coccidial activity (Nwosu et al., 2011), others were found to exert anti-coccidial function via immune action (Akhtar et al., 2012; Allen, 2003; Lee et al., 2011), suppression of oocyst wall formation (del Cacho et al., 2010), oocyst destruction (Remmal et al., 2011), anti-oxidant action (Allen et al., 1997, 1998; Naidoo et al., 2008; Orengo et al., 2012) and other mechanisms (Youn and Noh, 2001). Phytochemials, saponins and artemisinin have been proposed to be the active compounds against Coccidia (Allen et al., 1997; del Cacho et al., 2010; Mshvildadze et al.,

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2000). Despite these initial findings in early studies on anti-coccidial herbs, new anti-coccidial plants are still needed.

B. pilosa (Asteraceae) is an edible plant, commonly utilized as an ingredient in foods and medicines worldwide (Bartolome et al., 2013). The Food and Agriculture Organization of the United Nations advocated the cultivation of *B. pilosa* in Africa because of its high biosafety and easy growth (Young et al., 2010). Around 200 compounds have been identified from this plant including aliphatics, flavonoids, terpenoids, phenylpropanoids, aromatics, porphyrin and many others (Bartolome et al., 2013). The richness and complexity of the phytochemicals in *B. pilosa* may reflect the wide variety of bioactivities that have been reported for this herb, such as anti-microbial, anti-protozoal and many other actions (Bartolome et al., 2013). Nevertheless, the anti-coccidial properties of *B. pilosa* have not been evaluated.

In this study, batch consistency and quality control of a preparation of *B. pilosa* were assessed using phytochemical approaches, and the anti-coccidial activities of *B. pilosa* in chickens, as evidenced by survival rate, body weight loss, oocyst shedding and intestine pathology, were examined. Finally, the drug resistance of *B. pilosa* was evaluated.

2. Materials and methods

2.1. Plant preparation and analysis

The plant processing and analysis were performed similar to a previous publication (Chien et al., 2009). Three batches of the whole plant of *B. pilosa* were collected from Changhua County, Taiwan, and authenticated. After air drying at room temperature, the plant material was ground into a powder and the particles whose size ranges from 0.149 to 0.177 mm were collected for further use. For chemical fingerprint analysis, each batch of the pulverized *B. pilosa* material was extracted in 10-fold volumes of methanol at room temperature for 2 days. The crude extracts were evaporated by a rotary evaporator (Heidolph, Schwabach, Germany). After evaporation, the extracts were dissolved in water and subjected to high pressure liquid chromatography (HPLC) analysis using an RP-18 column (Phenomenex C18), hyphenated with a ultraviolet (UV) photodiode detector at 254 nm or a mass spectroscope (MS). The solvent gradient for HPLC was 0.1% TFA/acetonitrile (B) in 0.1% TFA/H_2O: 10–11% of B for 0–10 min, 11–19% of B for 10–15 min, 19–21% of B for 15 35 min, 21–28% of B for 35–47 min, and 28–100% of B for 47–55 min. Commercial standards, chlorogenic acid and isochlorogenic acid C were purchased from Sigma (St. Louis, MO, USA). The pulverized *B. pilosa* material from batch 1 was selected for the chicken diet formulation as described below.

2.2. Isolation, characterization and sporulation of E. tenella oocysts

Two isolates of *E. tenella* were collected from ceca of infected chickens after sacrifice at local poultry farms. Briefly, to obtain pure lines of *E. tenella*, different individual oocysts were sporulated with potassium dichromate and propagated throughout 2-week old chickens, one sporulated oocyst per chicken. Two isolates (Et C1, and Et C2) of *E. tenella* with ~20 μm in diameter were obtained and identified by microscopic method (Joyner and Long, 1974) and interspecies molecular characterization (Blake et al., 2008). All sporulated oocysts were maintained in the laboratory of the Department of Veterinary Medicine, National Chung-Hsing University for 3 years without exposure to any anti-coccidial drugs. The survival rate of the Lohmann chickens 7 days after challenge with Et C1 or Et C2 strain (1×10^4 sporulated oocysts) was ~60%. The Et C1 strain was used in this study unless indicated otherwise.

2.3. Animal husbandry, feed formulation and oral infection of E. tenella

In Experiment 1, 74 1-day-old disease-free Lohmann chicks from a local hatchery in Taichung, Taiwan, were obtained from a coccidian-free laboratory. To analyze the anti-coccidial action of *B. pilosa*, the chicks were randomly divided into six groups. There were four cages (4, 3, 3 and 3 chicks) in Group **1**, four cages (4, 4, 4 and 3 chicks) in Group **2**, four cages (4, 4, 4 and 3 chicks) in Group **3**, four cages (4, 3, 3 and 3 chicks) in Group **4**, three cages (3, 3 and 3 chicks) in Group **5,** and three cages (3, 3 and 3 chicks) in Group **6**. Chicks in all cages had *ad libitum* access to feeds and water throughout the experiment. Group **1** (UI control) and Group **2** (I control) had daily access to standard chicken diet (63.5% yellow corn, 16% soybean meal, 10% full fat soybean, 3.5% fish meal, 3% bran, 1.2% soybean oil, 1% calcium carbonate, 1.1% dicalcium phosphate, 0.4% salt, 0.2% lysine, 0.02% vitamin premix, 0.08% mineral premix) from day 1 to day 21. Group **3** (I Mad control) had daily access to the same diet supplemented with maduramicin (6 mg/kg diet). Group **4** (Bp5), Group **5** (Bp1), and Group **6** (Bp0.5) had daily access to the diet supplemented with *B. pilosa* powder at a dose of 5% (50 g/kg diet), 1% (10 g/kg diet) or 0.5% (5 g/kg diet), respectively. Chickens were inoculated on day 14. The chickens in Group **1** (UI control) were administered with 2 ml of phosphate buffered saline (PBS) and those in Groups **2** (I control), **3** (I Mad control), **4** (Bp5), **5** (Bp1) and **6** (Bp0.5) were infected with *E. tenella* sporulated oocysts (1×10^4). All animals were handled according to the guidelines of the National Chung Hsing University Institutional Animal Care and Use Committee (IACUC).

2.4. Measurement of survival rate, body weight, oocyst numbers, and gross and microscopic lesion scores in animals

Survival rate and bird appearance were checked daily. All birds in each cage in Experiment 1 were weighed on days 1, 7, 14 and 21 after hatching. Following published protocols in the literature (Conway et al., 1999; Haug et al., 2006), fecal samples were collected daily, from days 3 to 7 post infection, and weighed. Diluted oocyst suspension was prepared by adding water to 1 g of each fecal sample, followed by a serial filtration with W.S. Tyler sieves (1 mm, 250 μm and 45 μm). After centrifugation, the oocysts were suspended in saturated salt solution and mixed thoroughly. The homogenous suspension was transferred into two McMaster chambers for oocyst counts, with three repeats for each sample. Fecal oocyst number was calculated from the average of three counts of each sample. All the chickens in each group were sacrificed on day 21 and their ceca were removed. Gross lesion scores are obtained as described previously (Johnson and Reid, 1970). Briefly, gross lesions in the ceca caused by *E. tenella* were scored based on 5 grades: 0, normal tissue with no gross lesions; 1, very few scattered petechiae on cecal wall with normal cecal contents; 2, more numerous petechiae on thickened cecal wall with normal cecal contents; 3, noticeable cecal cores on greatly thickened cecal wall, large amounts of bloody cecal contents, and 4, greatly distended cecal wall with bloody or large caseous cores or dead birds. Microscopic lesion scores were obtained from the summation of lesion distribution and mucosal severity as published (Goodwin et al., 1998). Briefly, the entire ceca from the birds were fixed with 10% formalin and embedded in paraffin, followed by hematoxylin and eosin staining. The location of cecal lesions and mucosal histology were examined. The distribution of *E. tenella* infection along the observed cecal segment was graded as follows: 0, no *Eimeria* in any microscopic field at 10-fold magnification; 1, *Eimeria* in one field; 2, *Eimeria* in two fields; 3, *Eimeria* in three fields and 4, *Eimeria* in all four fields. The severity score in mucosae was graded as follows: 0, *Eimeria* in 0% of villi; 1, *Eimeria* in < 25% of villi; 2, *Eimeria* in 25 to 50% of villi; 3, *Eimeria*

in 51 to 75% of villi; 4, *Eimeria* in > 75% of villi. The microscopic lesion score is the sum of grades (0–4) found in five section slides per cecum.

2.5. Development and evaluation of drug resistance in E. tenella

In Experiment 2, 169 newly hatched chickens were purchased for drug resistance testing. Drug resistance of *E. tenella* in chickens was induced according to a previously described protocol with slight modification (Bafundo and Jeffers, 1990; Chapman, 1984). Briefly, *E. tenella* was passaged in chickens fed standard diet alone or supplemented with *B. pilosa* (0.5%) from day 0 to day 21 to obtain the first-generation oocysts. Such passage continued until the fifth-generation *Eiemria* oocysts were produced. Similarly, *E. tenella* was passaged in chickens fed a standard diet supplemented with salinomycin (70 ppm) from day 12 to day 21 until the fifth-generation oocysts were obtained. The above three lines were passaged in chickens fed a standard diet alone or supplemented with *B. pilosa* (1%) and salinomycin (140 ppm) for another three rounds, respectively. To assess drug resistance of the lines after eight serial passages, the three *Eimeria* lines were used to infect chickens in Groups **8**, **9** and **10** on day 14. Groups **7** (10 chickens, UI control) and **8** (10 chickens, I control) had daily access to standard chicken diet from day 1 to day 21. Groups **9** (15 chickens, Bp1) was fed daily with the diet supplemented with *B. pilosa* at the dose of 1% (10 g/kg diet) from day 1 to day 21. Group **10** (10 chickens, I Salino control) was given the diet supplemented with salinomycin (140 ppm) from day 12 to day 21. Drug resistance of *E. tenella* in the above experiments were assessed by the anti-coccidial index (ACI) based on the following formula (Li et al., 2004; Wang et al., 2006): ACI = [relative body weight gain (RBWG, %) + survival rate (SR, %)] − [lesion score index (LSI) + oocyst count index (OI)], where ACI ≥160 is defined as sensitive to the anti-coccidial drug, ACI between 120 and 160 is partially resistant to the anti-coccidial drug, and ACI < 120 is resistant to the anti-coccidial drug. RBWG = (100 × BWG per group)/[BWG of the uninfected unmedicated group (Group **7**, UI control)]; SR = (100 × the number of living chickens)/(total number of chickens per group); LSI = 10 × (lesion score per group); and OI = 100 × 0.4 × (oocyst counts per group)/[oocyst counts for unmedicated-infected group (Group **8**, I control)].

2.6. Statistical analysis

Data from nine chickens or more in each group of chickens in Experiments 1 and 2 are presented as mean ± standard error (SE). The cage in each group was used as the experimental unit. Pearson's chi-square test was used to determine whether there was a significant difference in the survival rate between treatment groups and control groups. Data on weight gain were subjected to two way ANOVA with factors group and cage (group) using the GLM procedure of SAS system. The excreted oocyst values were transformed into $\ln(x+1)$ and, in turn, analyzed by ANOVA using the GLM procedure of SAS system under a normal distribution. Lesion scores were analyzed using a chi-square test after multinomial transformation. Actual *P* values are presented in all experiments.

3. Results

3.1. Chemical fingerprinting techniques for assessment of batch consistency and quality control of B. pilosa added to chicken diets

Batch consistency and quality control of *B. pilosa* in different preparations is important for the success of applications of *B. pilosa* products in chicken diseases. Therefore, we first employed high-performance liquid chromatography (HPLC), ultraviolet (UV) spectroscopy and mass spectroscopy to analyze the chemical fingerprints of three batches of *B. pilosa* preparations. Each preparation was made from a different plant sample. The HPLC profiles of the three batches of *B. pilosa* extracts were highly similar, suggesting good batch consistency among the *B. pilosa* preparations (Fig. 1A). The identity of two peaks, chlorogenic acid (**1**) and isochlorogenic acid C (**2**), in the extracts were confirmed using UV and mass spectroscopy compared with commercial standards (Fig. 1B and Supplementary Figs. S1 and S2). Both compounds can serve as index compounds for quality control of *B. pilosa* preparations. Overall, chemical fingerprint analyses confirmed batch consistency and quality control of *B. pilosa* preparations.

3.2. Effect of B. pilosa on survival rate of chickens following E. tenella challenge

To examine the anti-coccidial effect of *B. pilosa* as a feed additive on chickens, chickens were given daily access (day 1–21) to standard chicken feed or feed containing maduramicin or *B. pilosa* powder (at doses of 0.5%, 1% and 5% of chick feed) (Fig. 2A). The survival rate of chickens with access to standard feed dropped from 100% (Group **1**; UI control) to 60% (Group **2;** I control) after *E. tenella* infection (Fig. 2B). As expected, the survival rate of infected chickens with access to feed containing maduramicin was 93% in Group **3** (I Mad control, Fig. 2B). In contrast, the survival rate was 100% for the infected chickens with access to feed containing 0.5% or more *B. pilosa* (Groups **4** (Bp5), **5** (Bp1) and **6** (Bp0.5), Fig. 2B). Furthermore, we examined the anti-coccidial effect of *B. pilosa* on challenge with a mixture of *E. tenella*, *E. maxima* and *E. acervulina*. We found that *B. pilosa* significantly increased the survival rate of infected chickens (Supplementary Fig. S4).

3.3. Effect of B. pilosa on reduced weight gain of chickens following E. tenella challenge

Next, we monitored body weight of chickens with access to different diets before and after *Eimeria* infection. Instead of using repeated measurement, we used the change in body weight of the chickens as a single measurement variable in the test in which individual chickens with similar initial weights were chosen. This method of measurement avoided time dependent confounding. The body weight gain in chickens of each group from day 21 and day 14 to day 1 is presented in Table 1. Two-way nested ANOVA with factors group and cage (group) was used to compare the body weight gain data. The actual *P* values are indicated in Table 1. There was no significant difference between each cage in each group.

On day 14, there was no significant difference in the body weight gain of the chickens in Group **3** (I Mad control), Group **4** (Bp5) and Group **5** (Bp1) in comparison with Group **1** (UI control) and Group **2** (I control). In contrast, the body weight gain in chickens of Group **6** (Bp0.5) was significantly different from those of the chickens in Group **1** (UI control) and Group **2** (I control). On day 21, there was a significant difference in the body weight gain of the chickens in Groups **3** (I Mad control), Group **4** (Bp5), Group **5** (Bp1) and Group **6** (Bp0.5) in comparison with Group **1** (UI control) and Group **2** (I control). Overall, *B. pilosa* significantly ameliorated reduced weight gain caused by *E. tenella* to a greater degree than maduramicin or control feed alone. Part of this amelioration may be attributed to the weight-gaining effect of *B. pilosa*.

3.4. Effect of B. pilosa on fecal oocyst excretion of chickens following E. tenella challenge

To further determine the anti-coccidial effect of *B. pilosa* in chickens, *Eimeria* oocysts in chicken feces, an indicator of *Eimeria* multiplication, was evaluated. No fecal oocysts were detected in the

Table 1
Body weight gain (BWG) of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 7 days post *E. tenella* infection.

Group[a] cage no. (chickens)	BWG (g)[b]	*P* value[c]	*P* value[d]	BWG (g)[b]	*P* value[c]	*P* value[d]
	Day 14 – 1	Day 14 – 1	Day 14 – 1	Day 21 – 1	Day 21 – 1	Day 21 – 1
1 (*n* = 13) 4(4, 3, 3, 3)	75.0 ± 5.1			145.4 ± 5.5		
2 (*n* = 15) 4(4, 4, 4, 3)	76.1 ± 3.9	>0.05		111.7 ± 5.5	<0.0001	
3 (*n* = 15) 4(4, 4, 4, 3)	77.9 ± 4.0	>0.05	>0.05	120.1 ± 7.9	<0.0001	0.008
4 (*n* = 13) 4(4, 3, 3, 3)	79.0 ± 4.2	>0.05	>0.05	121.1 ± 2.9	<0.0001	<0.0001
5 (*n* = 9) 3(3, 3, 3)	85.9 ± 12.0	>0.05	>0.05	121.8 ± 5.7	<0.0001	0.0023
6 (*n* = 9) 3(3, 3, 3)	86.1 ± 3.9	0.00095	<0.0001	134.5 ± 5.9	<0.0001	<0.0001

[a] The chickens were classified into six groups. Group 1 (UI control) and Group 2 (I control) had daily access to standard chicken diet from day 1 to day 21. Group 3 (I Mad control) were daily given the diet supplemented with maduramicin (6 mg/kg diet). Group 4 (Bp5), Group 5 (Bp1), and Group 6 (Bp0.5) were daily fed with the diet supplemented with *B. pilosa* powder at the dose of 5% (50 g/kg diet), 1% (10 g/kg diet) or 0.5% (5 g/kg diet), respectively. The number (*n*) of chickens in each group, cage number in each group and chicken number in each cage are indicated.

[b] Body weight gain (BWG) was obtained by the formula: body weight on day T (14 or 21) – body weight on day 1.

[c] The difference in body weight gain (g) of the chickens between infected groups (Groups **2**–**6**) and uninfected unmedicated group (Group **1**) is analyzed by nested ANOVA and shown by *P* value.

[d] The difference in body weight of the chickens between the infected medicated groups (Groups **3**–**6**) and infected unmedicated group (Group **2**) is analyzed by nested ANOVA and shown by *P* value.

uninfected unmedicated controls (Group **1** (UI control), Table 2). Fecal oocyst excretion was first detected on day 4 post-infection in all *E. tenella*-infected groups and peaked on day 7 post-infection (Group **2** (I control), Table 2). As expected, the infected maduramicin-fed birds in Group 3 (I Mad control, Table 2) had significantly fewer oocysts per gram of feces than the infected controls in Group 2 (I control, Table 2). Similarly, the infected *B. pilosa* diet-fed birds in Group 4 (Bp5) and Group 5 (Bp1) and Group **6** (Bp0.5) had significantly fewer oocysts per gram of feces than those in Group 2 (I control) as shown in Table 2.

3.5. *Effect of* B. pilosa *on intestinal lesions of chickens following* E. tenella *challenge*

Next, gross examination of the cecum in the animals that had access to different diets was performed 7 days after *Eimeria* infection. The gross cecal lesion score is shown in Table 3. The uninfected unmedicated control chickens (Group **1** (UI control), Table 3) had no lesions in the ceca (score = 0). In contrast, *E. tenella* caused more gross cecal lesions in the gut of unmedicated chickens 7 days post-infection, as evidenced by a lesion score close to 4 (Group **2** (I control), Table 3). Like maduramicin (Group **3** (I Mad control)), *B. pilosa* at different doses (0.5%, 1% and 5%) significantly diminished cecal damage in infected chickens (Groups **4** (Bp5), **5** (Bp1) and **6** (Bp0.5), Table 3) as shown by the gross lesion scores of 2–3. Mucosal damage caused by coccidia was examined by microscope and scored as microscopic cecal lesions based on the distribution and severity of mucosal destruction in chicken cecum (Table 4). No microscopic cecal lesions (score = 0) were observed in the uninfected unmedicated control group (Group **1** (UI control), Table 4), akin to the observation for gross cecal lesions in the same animals (Group **1** (UI control), Table 3). In sharp contrast, the infected unmedicated animals showed serious microscopic lesions (score = 7.8) in ceca 7 days after *Eimeria* infection (Group **2** (I control), Table 4). Severe ulceration, hemorrhage and decreased villi were also observed in ceca (data not shown). Oocysts, gametocytes and schizonts appeared inside the cecal epithelia (data not shown). The infected maduramicin-fed animals (Group **3** (I Mad control), Table 4) showed mild improvement in microscopic lesions (score = 7.3) in the cecum from the infected unmedicated animals (Group **2** (I control), Table 4) post infection. However, the infected *B. pilosa* diet-fed animals (Groups **4** (Bp5), **5** (Bp1) and **6** (Bp0.5), Table 4) showed significantly reduced microscopic lesions (scores of 1.0–1.7) in the cecum. Consistently, *B. pilosa* decreased ulceration and hemorrhage and preserved more mucosae and villi in chicken ceca than control diets (data not shown). *B. pilosa* also decreased the number of oocysts, gametocytes and schizonts inside the cecal epithelia to a greater extent than maduramicin and control diets (data not shown). Overall, *B. pilosa* significantly reduced gut pathology in chickens following *E. tenella* infection.

Table 2
Fecal oocyst excretion of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 3–7 days post *E. tenella* infection.

Group	Days post-infection				
	3	4	5	6	7
	Ln(OPG + 1)	Ln(OPG + 1)	Ln(OPG + 1)	Ln(OPG + 1)	Ln(OPG + 1)
1 (*n* = 13)	0	0	0	0	0
2 (*n* = 15)	0	11.44 ± 0.11[a]	12.18 ± 0.07[a]	13.39 ± 0.11[a]	13.96 ± 0.05[a]
3 (*n* = 15)	0	11.09 ± 0.05[a,b]	11.93 ± 0.09[a,b]	13.04 ± 0.06[a,b]	13.66 ± 0.04[a,b]
4 (*n* = 13)	0	10.53 ± 0.12[a,b]	11.83 ± 0.09[a,b]	12.87 ± 0.06[a,b]	13.55 ± 0.11[a,b]
5 (*n* = 9)	0	10.80 ± 0.07[a,b]	11.72 ± 0.09[a,b]	13.01 ± 0.03[a,b]	13.58 ± 0.02[a,b]
6 (*n* = 9)	0	9.55 ± 0.40[a,b]	11.54 ± 0.16[a,b]	12.87 ± 0.10[a,b]	13.21 ± 0.09[a,b]

The oocysts per gram feces (OPG) of the same chickens from Table 1 in Experiment 1 were counted from day 3 to day 7 post infection. The OPG values ($\times 10^4$) of the chickens in each group were transformed into Ln(OPG + 1) and analyzed with ANOVA using the GLM procedure of SAS system under a normal distribution. The number (*n*) of chickens in each group is indicated.

[a] The difference in OPG in the chickens between the infected groups (Groups **2**–**6**) and uninfected unmedicated group (Group **1**) on the indicated days is statistically significant with a *P* value < 0.05.

[b] The difference in OPG in the chickens between the infected medicated groups (Groups **3**–**6**) and infected unmedicated group (Group **2**) on the indicated days is statistically significant with a *P* value < 0.05.

Table 3
Gross lesion scores in the ceca of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 7 days post *E. tenella* infection.

Group[b]	Gross lesion score[a]					Average[b]	*P* value[c]
	0	1	2	3	4		
1 ($n = 13$)	13	0	0	0	0	0.0 ± 0.0	
2 ($n = 15$)	0	0	0	4	11	3.7 ± 0.4	
3 ($n = 15$)	0	0	3	6	6	3.2 ± 0.8	0.0876
4 ($n = 13$)	0	0	5	5	3	2.8 ± 0.8	0.0003
5 ($n = 9$)	0	1	3	4	1	2.6 ± 0.9	0.0091
6 ($n = 9$)	0	1	5	3	0	2.2 ± 0.7	0.0008

[a] Gross lesion of the ceca of the same chickens from Table 1 in Experiment 1 was examined and scored as described in the Materials and methods section. The numbers represent the number of chickens with cecal gross lesions in five grading categories (0–4) and the number (n) of total chickens in each group, respectively.
[b] Average is expressed as the least square mean ± SE.
[c] The difference in the gross lesions of the ceca of chickens between the infected medicated groups (Groups **3**–**6**) and infected unmedicated group (Group **2**) is analyzed by chi-square test after multinomial transformation and shown by *P* value.

3.6. *Drug resistance of* E. tenella *to* B. pilosa *in chickens*

In parallel, we tested drug resistance of *E. tenella* to *B. pilosa* using the ACI, which is a commonly-used index for the assessment of drug resistance (Li et al., 2004; Wang et al., 2006). Weight gain, survival rate, fecal oocyst excretion and lesion scores of the four groups of experimental chickens (Table 5) were used to calculate this index. After eight passages in infected chickens given standard diet for 168 days, the ACI value was 200 and 47, respectively, for the uninfected unmedicated chickens (Group **7** (UI control), Table 5) and infected unmedicated chickens (Group **8** (I control), Table 5). In contrast, the ACI value was 146 and 40, respectively, for chickens given 1% *B. pilosa* (Group **9** (Bp1), Table 5) and 140 ppm salinomycin (Group **10** (I Salino control), Table 5), indicating the high drug resistance to *E. tenella* induced by salinomycin but not *B. pilosa*. The overall data demonstrated that long-term use of 1% *B. pilosa* showed low drug resistance to *E. tenella*, a superior result to that for salinomycin.

4. Discussion

Avian coccidiosis poses a continuous challenge to the poultry industry. Due to unmet efficacy and side effects of anti-coccidial drugs and vaccines, edible plants are considered possible viable alternative substituents to replace current anti-coccidial approaches (Orengo et al., 2012; Remmal et al., 2011). Here, we established spectroscopic methods for chemistry, manufacturing and control of *B. pilosa* (Fig. 1 and Supplementary Figs. S1 and S2). We also demonstrated that *B. pilosa* protects chickens against *Eimeria* infection (Fig. 2 and Tables 1–4) and resistance of *E. tenella* is poorly developed after long-term treatment with *B. pilosa* (Table 5). This study, for the first time, proved the feasibility of the use of *B. pilosa* as an anti-coccidial agent in chickens.

Chicken *E. tenella* infection rate and mortality are 20–100% and 20–60%, respectively. The severity of both indices is dependent on chicken genetics and *Eimeria* species (Abu-Akkada and Awad, 2012). For example, the mortality of chickens caused by different isolates of *E. tenella* can reach up to 40% (Dakpogan et al., 2012). In our study, *E. tenella* isolate, Et C1, was isolated and amplified from a single *E. tenella* oocyst in chickens. This *E. tenella* isolate was considered to be a pure strain based on its morphological traits and molecular markers (Supplementary Fig. S3). We found that *E. tenella* used in this study caused ~40% of chicken death (Fig. 2B). Clearly, this high mortality is attributable to the virulence, but not the impurity, of the *E. tenella* isolate.

So far, 20 or so plants have been shown to possess anti-coccidial activities. Nevertheless, some of them showed discrepancies in *in vitro* and *in vivo* anti-protozoal bioactivities (van der Heijden and Landman, 2008a, 2008b). One explanation could be lack or insufficiency of batch consistency and/or quality control in the preparation of the plant products. In this work, we established a protocol by which to prepare and analyze *B. pilosa* extracts using phytochemical techniques (Fig. 1 and Supplementary Figs. S1 and S2). These efforts can ensure the quality of *B. pilosa* as an anti-coccidial formulation.

More importantly, our data showed that *B. pilosa* is prophylactically effective against *E. tenella* in young chickens aged 14 days (Fig. 2 and Tables 1–4). Survival rate, body weight, oocyst shedding, and cecal lesions were used as indicators by which to evaluate the anti-coccidial potential and drug resistance to *E. tenella* of *B. pilosa* using the same protocols as published elsewhere (Awais et al., 2011;

Table 5
Evaluation of drug resistance of *E. tenella* after eight serial passages in chickens given salinomycin and *B. pilosa*.

Group	RBWG (%)	SR (%)	LSI	OI	ACI
7 ($n = 10$)	100	100	0	0	200
8 ($n = 10$)	60.3	60	33.8	40	46.6
9 ($n = 15$)	79.5	100	23.3	10	146.2
10 ($n = 10$)	32.4	80	32.5	40	39.9

In Experiment 2, experimental induction and assessment of drug resistance of *E. tenella* in Groups **7** (uninfected unmedicated chickens, UI control), **8** (infected unmedicated chickens, I control), **9** (infected *B. pilosa*-fed chickens, Bp1) and **10** (infected salinomycin-fed chickens, I Salino control) are described in the Materials and methods section. The formulae for the RBWG, SR, LSI, OI and ACI values are also indicated in the Materials and methods section. The number (n) of chickens in each group is indicated.

Table 4
Microscopic lesion scores in the ceca of chickens fed standard diet alone or supplemented with maduramicin and different doses of *B. pilosa* 7 days post *E. tenella* infection.

Group	Microscopic lesion score[a]									Average[b]	*P* value[c]	
	0	1	2	3	4	5	6	7	8		*Pa*	*Pb*
1 ($n = 13$)	65	0	0	0	0	0	0	0	0	0.0 ± 0.0		
2 ($n = 15$)	0	0	0	0	0	0	1	6	68	7.8 ± 0.4	<0.0001	
3 ($n = 15$)	0	0	0	2	0	4	10	7	52	7.3 ± 1.2	<0.0001	0.0036
4 ($n = 13$)	23	0	24	7	11	0	0	0	0	1.7 ± 1.5	<0.0001	<0.0001
5 ($n = 9$)	16	0	19	2	8	0	0	0	0	1.7 ± 1.5	<0.0001	<0.0001
6 ($n = 9$)	22	0	23	0	0	0	0	0	0	1.0 ± 1.0	<0.0001	<0.0001

[a] Microscopic lesion of the ceca of the same chickens from Table 1 in Experiment 1 was examined and scored as described in the Materials and methods section. The number represent the sum of microscopic lesions (0–4) in the gut samples, five section slides per gut, and the number of the examined gut samples multiplied by 5 per group.
[b] Average = Least square means ± SE.
[c] The difference in microscopic lesion scores of the chickens between infected medicated groups and uninfected unmedicated group (Group **1**) is analyzed with a chi-square test after multinomial transformation and shown by *Pa* value. Similarly, the difference in microscopic lesion scores of the chickens between infected medicated groups (Group **3–6**) and infected unmedicated group (Group **2**) is shown by *Pb* value.

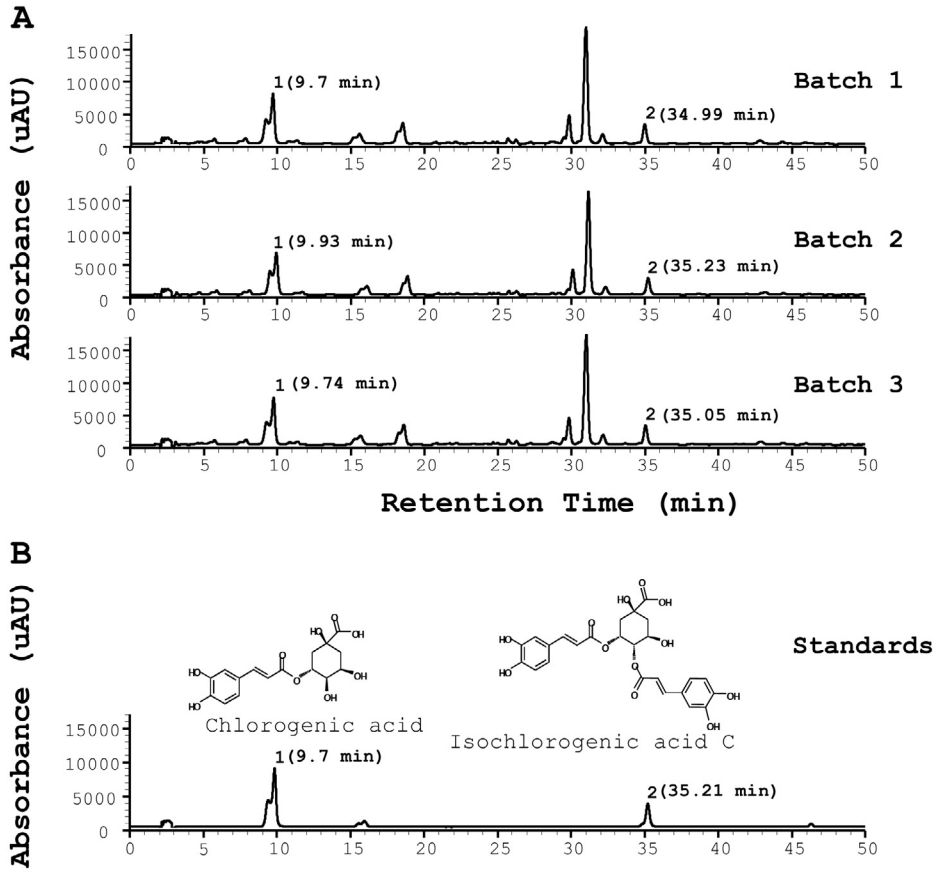

Fig. 1. HPLC chromatograms of *B. pilosa* extracts and chlorogenic acid standards. (A) Three batches of *B. pilosa* extracts were prepared. The extracts underwent HPLC chromatography with UV photodiode detection at 330 nm. Chlorogenic acid (**1**) and isochlorogenic acid (**2**) were identified as index compounds in these extracts. (B) HPLC profile of the mixture of commercial standards, chlorogenic acid (**1**) and isochlorogenic acid C (**2**). Retention time of peaks **1** and **2** in different batches of *B. pilosa* extracts and standards is indicated in the parentheses.

Hassan et al., 2008). Our study demonstrated that *B. pilosa* at the dose of 0.5% of chicken feed or more, conferred 100% protection against *Eimeria* challenge in chickens (Fig. 2B). *B. pilosa* consistently reduced fecal oocyst excretion (Table 2) and degree of intestine destruction (Table 3 and 4). Accordingly, *B. pilosa* treatment improved the reduced weight gain in chickens infected with *Eimeria* (Table 1). This improvement can be attributed to the ability of *B. pilosa* to control *Eimeria* infection and, to some degree, to induce gain weight in chickens (Table 1). The modest weight-gaining effect of *B. pilosa* powder reflects the fact that this plant is used as a food and has nutritional value as described elsewhere (Bartolome et al., 2013). *B. pilosa* at 0.5% of chicken diet is effective against chicken coccidiosis in our experimental system. However, higher doses of *B. pilosa* seems bad for coccidiosis control as evidenced by weight gain, gut pathology and oocyst excretion. These detrimental effects may be associated with the higher viscosity of the gut content when more *B. pilosa* is added to the chicken diet.

Apart from anti-coccidial action, *B. pilosa* has several other advantages over the anticoccidial drugs, maduramicin and salinomycin. First, *B. pilosa* is an edible plant and, therefore, there is little concern about biosafety and drug residence in chicken meat. Second, *B. pilosa* has a novel anti-coccidial action, which is different from that of commercial drugs. Third, long-term use of *B. pilosa* shows much lower drug resistance than that of salinomycin. This application of *B. pilosa* may reduce massive use of anti-coccidial drugs, anti-coccidial drug residue in chicken products, generation of drug-resistant mutants and concerns about public health. This work also expands the medicinal utility of *B. pilosa* in veterinary medicine.

Drug resistance has been reported against almost all anticoccidial drugs and is a major issue for coccidiosis control (Li et al., 2005). The ACI values revealed that drug resistance of *E. tenella* to salinomycin significantly increased in 168-day induction experiments (Table 5). In sharp contrast, its drug resistance to *B. pilosa* was poorly developed (Table 5). Of note, the degree of the drug resistance to *B. pilosa* may be underestimated because the weight-gaining effect of this plant. Nevertheless, the ACI data suggest that *B. pilosa* induced little, if any, drug resistance. The reason for this may be that this plant possesses multiple bioactive compounds, which may simultaneously inhibit different pathways of *E. tenella*. It is not hard to image that *E. tenella* can develop drug resistance to one agent more easily than multiple agents with different chemical structures.

The anti-coccidial mechanism of action of *B. pilosa* is currently unclear and needs to be ascertained in further studies. Direct chemical destruction and attenuation of invasive sporozoites are the primary reasons for decreases in oocyst excretion, induction of precocious lines and control drug resistance (Li et al., 2004; McDonald and Shirley, 2009). Since *B. pilosa* significantly reduced the shedding of fecal oocysts (Table 2) and drug resistance (Table 5), it is plausible that the compounds in *B. pilosa* act to destroy and attenuate Coccidia. Our earlier publications showed that *B. pilosa* can increase Th2 immunity (Chang et al., 2004, 2005), related to eradication of intestinal helminth, and inhibit the propagation of enteric bacteria (Chang et al., 2007a, 2007b). These findings suggest that *B. pilosa* may promote the clearance of Coccidia via immune regulation. In the future, identification of the active compounds in *B. pilosa*

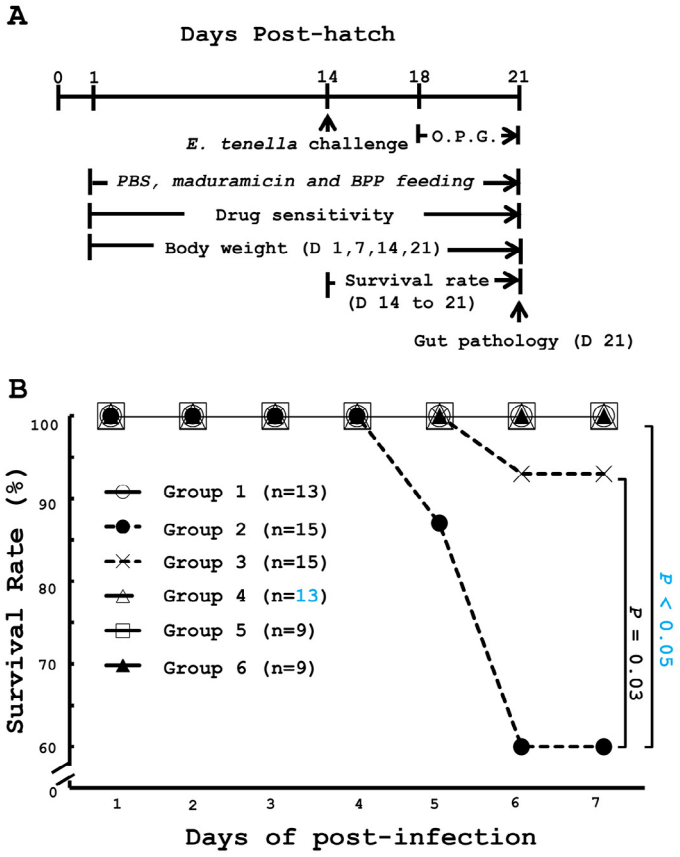

Fig. 2. *B. pilosa* increases survival rate of chickens given *E. tenella* challenge. (A) Summary of the experimental protocol of Experiment 1 in this study. (B) The same chickens as in Table 1 were divided into six groups. They had daily access to standard diet (Group **1** (UI control) and Group **2** (I control)) and the diet containing maduramicin (Group **3** (I Mad control)) or different doses of *B. pilosa* (Group **4** (Bp5), Group **5** (Bp1), and Group **6** (Bp0.5)). On day 14, chickens were infected with PBS or *E. tenella* sporulated oocysts (1×10^4) by gavage. Survival rate was monitored from day 1 to 7 post infection. The number (n) of chicks per group and P values are indicated.

that are active against coccidiosis will be pivotal for unveiling its mode of action.

5. Conclusions

Here, we performed chemical fingerprint analyses to determine batch consistency and quality control of *B. pilosa* preparations, and demonstrated that *B. pilosa*, used as a feed additive can protect against *E. tenella* in chickens by reducing mortality, oocyst excretion and intestinal lesions. In addition, *B. pilosa* decreased the induction of drug-resistant *E. tenella*. In summary, this study illustrates the anti-coccidial potential of *B. pilosa* in chickens.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rvsc.2014.11.002.

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