Research in Veterinary Science 98 (2015) 74–81

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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

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ARTICLE INFO

Article history:
Received 10 March 2014
Accepted 6 November 2014

Keywords:
Bidens pilosa
Eimeria
Coccidiosis
Chicken
Anti-coccidia

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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http://dx.doi.org/10.1016/j.rvsc.2014.11.002
0034-5288/

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*