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Evaluation of Selected Herbal Extracts on Clinicopathological Features and Therapeutic Efficacy in Experimental Avian Coccidiosis: *In Vitro* and *In Vivo* Approaches

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ABSTRACT

Background: Development of resistance to synthetic anticoccidial drug has triggered the search for safe alternatives for the therapeutic management of avian coccidiosis. Different herbal plants were reported to have potential characteristics against coccidiosis. The current study focused to explore innovative herbal remedy using native Bangladeshi plants for avian coccidiosis, aiming to offer alternative solutions for its treatment.

Methods: *In vitro* and *in vivo* anticoccidial properties of aqueous extracts of *Psidium guajava* leaves were assessed. *In vitro* oocyst counts, oocysticidal assays, etc. were conducted with oocysts from five locally isolated *Eimeria* species exposed to four different concentrations (0.25%, 0.50%, 0.75% and 1.0%) of *Psidium guajava* extracts. The *in vivo* anticoccidial efficacy of *Psidium guajava* leaf extracts like effects on clinical signs, mortality rate, intestinal lesion score, fecal scores, etc. were studied on 560 day-old broiler chicks at four different concentrations, while Amprolium (0.125%) serving as the standard anticoccidial drug.

Results: *Psidium guajava* leaves extracts showed significant improvement on clinicopathological manifestations and therapeutic efficacy in terms of clinical signs, mortality rate, fecal scores, intestinal lesion scores, oocyst counts, etc. in experimentally induced coccidiosis in chicken in a dose dependent manner ($p < 0.05$). Supplementation of 0.75% and 1.0% guava leaf extracts per liter drinking water yielded comparable therapeutic efficacy with synthetic anticoccidial. Mortality was reduced from 46.3% in infected group to 6.3% in 1.0% guava leaf extracts groups which didn't differ significantly from the mortality (5%) of the group treated with synthetic anticoccidial ($P>0.05$). There was no significant difference between synthetic anticoccidial drug and supplementation of 1.0% guava leaves extracts in oocysts reduction rate which were 92.7% and 94.6%, respectively ($p<0.05$).

Conclusion: Our study findings explored the potentials of *Psidium guajava* extract as an alternative to synthetic anticoccidials which could be used in the treatment and control of avian coccidiosis.

Keywords: Guava; *Eimeria*; Oocysts; Anticoccidial; Herbal

Abbreviations: DPI: Days of Post Infection; OPG: Oocysts Per Gram; ANOVA: Analyzed by Analysis of Variance; LSD: Least Significant Difference; SAURES: Sher-e-Bangla Agricultural University Research System; SI: Sporulation Inhibition

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Highlights

- Avian coccidiosis is an important economic disease that brings huge financial loss to the poultry industry of Bangladesh.
- Synthetic anticoccidials have constraints like development of resistance and cost effectiveness.
- Herbal medicine can be a safe alternative to synthetic compounds for the treatment and control of disease in poultry.
- *Psidium guajava* (leaves) extracts has promising effects for controlling of coccidiosis in broiler chickens.

Introduction

The poultry industry is an emerging and significant sector that has been making progressive contributions to Bangladesh's economy over the past decade. It is one of the fastest growing and most promising industries, with a bright future for the country [1]. The poultry sector is vital in reducing poverty, malnutrition, and unemployment problems in the country. Poultry plays a vital role in human livelihood by contributing to the food supply, generating income and employment, and supplying raw materials to various industries [2]. Thus, the poultry sub-sector is an important avenue to reduce Bangladesh's protein deficiency, poverty, and high unemployment problems. However, this promising industry often encounters different challenges that hinder its growth and development. Different diseases affecting poultry are thought to be the most important obstacle in modern commercial and backyard poultry production in Bangladesh [3]. Avian coccidiosis is one of the most important economic diseases of poultry which is responsible for a significant mortality rate in broiler [4]. Coccidiosis is ubiquitous in all types of broiler farming. Thus, it has been recognized as the parasitic disease that has the greatest economic impact on poultry industries worldwide due to production losses and costs for treatment or prevention [4,5].

A recent study reported that the chicken industry of the US was burdened by about $127 million annually and similar losses are assumed to occur in other parts of the world [6]. Coccidiosis is likely the most costly and widespread infectious disease in commercial poultry systems. While chemoprophylaxis and anticoccidial feed additives have traditionally controlled the disease, the emergence of drug resistance and their potential toxic effects on animal health complicate the situation [7]. Additionally, drug or antibiotic residues in poultry products may pose risks to consumers [8]. Another method for controlling coccidiosis is vaccinating birds with live *Eimeria* oocysts, but poor management can lead to severe reactions, particularly affecting broiler performance due to their short rearing period [9]. To address these issues, attenuated vaccines have been developed, but their production is expensive [9]. Consequently, more cost-effective and safer alternatives for controlling avian coccidiosis are being explored [10,11]. Botanicals offer a promising alternative, as they are natural and may contain new therapeutic compounds to which resistance has not yet developed. Numerous botanicals, herbal complexes, and commercial herbal anticoccidials with promising effects have been documented in the literature [12].

At the same time, recently consumers have growing interest on complementary and alternative medicines, including herbal medicine, as they assume these forms of remedies as being both safe and effective [8]. The growing use of alternative and complementary healthcare has led scientists to explore the various biological activities of medicinal plants. While the efficacy and mechanisms of action of many of these plants have not been scientifically validated, their simple preparations often produce positive effects due to their active chemical components [13]. Plant-derived products are rich in a wide range of phytochemicals, including phenolic acids, flavonoids, tannins, lignin, and other small compounds [14]. These compounds exhibit numerous health benefits, such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic, and vasodilatory properties [15]. Herbal medicine has long been acknowledged as one of the oldest forms of treatment used by humans [16]. The therapeutic benefits of various medicinal plants have been applied to treat both human and animal diseases for centuries [17,18].

Despite advancements in modern medicine, many people in developing countries still depend on traditional healing methods and medicinal plants for their healthcare needs [19]. Research conducted in Africa, Asia, Europe, Latin America, and North America demonstrates that plants are commonly used to treat animal diseases [16,20-22]. Historically, it has been recorded that humans often use the same herbal remedies for their sick animals [15]. The exploration of herbal materials as anticoccidial remedies presents a promising alternative for controlling coccidiosis. Besides potentially reducing food production costs in Bangladesh, it could also foster an herbal remedy export market, generating more employment opportunities in the country. Moreover, the use of herbal extracts may address growing consumer concerns, provided they are proven to be both safe and effective. Although studies on the anticoccidial properties of available herbal medicine are being extensively conducted in many countries based on their native herbs, surprisingly, there is no or little documented information about the locally available herbs and their anticoccidial potentials in Bangladesh. No comprehensive study has yet been documented that reported the anticoccidial potential of Bangladeshi herbs though the country is a blessed kid of nature harboring plenty of herbs in her land.

Recently, considering the huge potential of herbal medicine, higher priority has been given to the 'Development of herbal drugs and treatment' in 'Agricultural Research Priority: Vision- 2030 and beyond' under the Livestock Sub-sector of Bangladesh [23]. Therefore, to fulfill the priority-based present research need in Bangladesh, this study aimed at the development of herbal anticoccidial as the alternative to synthetic anticoccidial against pathogenic avian coccidian species and thus to develop an alternative approach for the treatment and control of avian coccidiosis using herbal plants of Bangladesh.

Materials and Methods

Collection and Preservation of Plant Material

A comprehensive literature review was performed on the ethnoveterinary use of herbal plants in the Indo-Bangla Subcontinent. Based on this review and considering their availability in Bangladesh, guava was chosen for this study. Leaf samples were gathered from guava trees in various regions of Bangladesh. These samples were randomly collected, placed in plastic zip-lock bags with appropriate labels, and kept in an ice cooler until they were taken to the laboratory for extraction. An expert from the Department of Crop Botany at Sher-e-Bangla Agricultural University in Dhaka verified the authenticity of the collected fresh leaves. The leaves were then washed in a salt solution to remove any microbial contaminants, rinsed with clean water, and left to dry in the shade at room temperature. The dried leaves were crushed into a coarse extract using an electric grinder, dried again in the shade, and then ground into a fine powder. The resulting fine extracts was stored in nylon bags until needed for use.

Preparation of Plant Extracts

The plant's aqueous extracts were assessed for anticoccidial properties. For the aqueous extraction, 10 grams of air-dried guava leaf extracts were added to 250 milliliters of distilled water and boiled for 10 minutes. The solution was then filtered and subjected to low-speed centrifugation to obtain a supernatant free of debris and coarse particles. The aqueous extract was prepared using the methods outlined by Parekh et al., 2005 [24].

Experimental Drug

Amprolium, a commercially available water-soluble anticoccidial drug used for the routine treatment of avian coccidiosis caused by *Eimeria* species, was utilized to compare the anticoccidial effects of the plant powders.

Eimeria spp. Strains

The challenge oocysts were isolated from the intestines and ceca of naturally infected chickens, using sieving and sedimentation techniques [25]. The collected oocysts were left to sporulate at room temperature in a 2.5% potassium dichromate solution. Sporulated *Eimeria oocysts* were then cleared and counted per 1.0 ml of solution using the McMaster technique, as outlined by Soulsby [25]. The field strains of sporulated *Eimeria oocysts* were stored according to standard protocol until needed for *in vitro* and *in vivo* studies. The oocyst mixture used comprised 30% *E. tenella*, 20% *E. maxima*, 20% *E. acervulina*, 15% *E. necatrix*, and 15% *E. mitis*.

Experimental Birds

A total of 560 one-day-old unsexed Cobb 500 broiler chicks were sourced from a local commercial broiler supplier for the trial. The birds were raised under strict hygienic conditions, with feed and drinking water provided ad libitum. They were fed a balanced commercial diet free from anticoccidials. During the experiment, the chicks were floor-reared in separate units, and their feces were microscopically examined to confirm they were free from coccidiosis.

In vitro Study of Herbal Anticoccidials

Various concentrations of herbal plant extracts were prepared (0.25%, 0.5%, 0.75%, and 1.0%). For comparison, Amprolium was used at the recommended concentration as a reference drug. Separate Petri dishes containing 2 ml of each plant extract concentration were inoculated with an equal number of viable sporulated oocysts, incubated at 25 °C, and examined after 1 hour, 6 hours, 12 hours, and 18 hours. Each concentration was tested in three replications. The sporulation process of *Eimeria* oocysts was observed under a light microscope at 40x magnification. Sporulation inhibition (SI) and oocyst damage were calculated as a percentage, following the method outlined by Nghonjuyi et al. 2015 [26].

In Vitro Oocysticidal Effects of Plant Extracts

Sterile Petri dishes were used to assess the *in vitro* oocysticidal effects of experimental plant extracts. An aliquot of 2 ml of different extract concentrations (0.25%, 0.5%, 0.75%, and 1.0%) was poured in each well of the Petri dishes and inoculated with an equal number of unsporulated oocysts. The Petri dishes were incubated at 28°C. Phenol was used as a reference disinfectant for comparison. Observations were made after 24 and 48 hours. The number of sporulated and non-sporulated oocysts was counted, and the sporulation percentage was determined by counting the sporulated oocysts out of a total of 100 oocysts. The percentage of sporulation inhibition was calculated as follows:

$$\text{Sporulation inhibition\%} = \frac{Sp\ \%\ \text{of control-Sp\% of extract}}{Sp\ \%\ \text{of control}} X100$$

In Vivo Study of Herbal Anticoccidials

Experimental Design: A total of 560 day-old Cobb500 broiler chicks were randomly divided at 1st day of age into seven equal groups (from I to VII), each of 80 chicks, the groups were arranged as the following:

- Group I: Infected and supplemented with plant extract@ 0.25% (2.5 g/liter) in drinking water.
- Group II: Infected and supplemented with plant extract@ 0.5% (5 g/liter) in drinking water.
- Group III: Infected and supplemented with plant extract@ 0.75% (7.5 g/liter) in drinking water.
- Group IV: Infected and supplemented with plant extract @ 1.0% (10 g/liter) in drinking water
- Group V: Infected and treated with Amprolium@0.125% (1.25 g/liter) in drinking water.
    - Group VI: Infected - non-treated (isolated control positive).

- Group VII: Non-infected – non-treated (control negative).

All groups except group 7 were infected with 10,000 sporulated oocysts/bird orally of mixed *Eimeria spp*. at 7th day of age. Treatment was started on the same day of infection in drinking water (8 hrs. per day after thirst for one hour) till the end of the experiment (14th day of age). The preliminary acute toxicity study of the plant extract was conducted by using 20, day-old broiler chickens which were divided into four groups of five chickens each. Each bird in groups I-IV was individually drenched with the graded doses of the aqueous extract of *Psidium guajava* to be tested. The chickens were observed for 24 h for any signs of toxicity including change in behavior or death.

Clinical Examination and Mortality Rate: Clinical signs like inappetence, drowsiness, depression, ruffled feathers, bloody diarrhea, and death etc. were recorded. The mortality rate was determined using the following formula:

$$\%mortality = \frac{Total\ \text{no. of chicks in group}}{\text{Initial no. of birds in group}} X100$$

The mortality rate after being exposed to the infectious agent in the absence and presence of the herbal extract were calculated accordingly. After the onset of clinical signs, fecal scoring was conducted following the methods previously described elsewhere [27,28]. Bloody/tan diarrhea was graded from "-" to "+ + +" where "-" was assigned to the normal feces. Grades +, + +, and + + + corresponded to 33%, 33–66%, and 66– 99 % blood/dark color in total faces, respectively.

Effects of Plant Extracts on Oocysts Count: Freshly voided droppings were collected from each group for oocysts count daily from the 5th till the 10th day of post-infection (dpi). The samples were either processed or refrigerated for a maximum of 1 day until processing. Mean oocyst count per gram of feces for each group was recorded using a modified Mac Master Technique, following standard procedure [29]. Briefly, 2 grams of feces were mixed with 30 ml of a saturated salt solution and homogenized thoroughly. A portion of the solution was extracted immediately after mixing with a pipette and placed in one half of a Mac Master slide. Same procedure was repeated for the other half of the slide. The total oocysts count from both sides of the chamber was multiplied by 50 to estimate the number of eggs in the sample, with results reported as oocysts per gram (OPG).

Postmortem Examination: Post-mortem examination was conducted on the 5th, 7th, 9th, and 11th days post-infection. Three birds from each group were selected at random for post-mortem examination, along with all the birds that died during the experiment. The intestine and ceca of each bird were macroscopically examined to determine lesion scores. Lesion scores were graded into 4 grades (0 to III). If no evident lesions were detected, the lesion score was recorded as 0, while a score of III indicated severe affliction in the bird.

Statistical Analysis: The data obtained were statistically analyzed by analysis of variance (ANOVA) and comparing groups was performed using the least significant difference (LSD) at $p \leq 0.05$ according to Petrie and Watson, 1999 and computerized using SPSS 16.0.

Results

A total of 275.32 gm (23.52% w/w) aqueous leaf extract of *P. guajava* was obtained in this study. The initial acute toxicity test showed no indications of toxicity (such as salivation, drooling, panting, lethargy, eye blinking, or lack of coordination) or mortality in any of the treated broiler chicken groups, even those given the highest dose of *P. guajava* aqueous leaf extract.

Effects of Aqueous Leaf Extract of *p. guajava* on Clinical Signs

Figure 1 illustrates the clinical signs manifested by the birds in which experimental coccidiosis was induced with field isolates. Bloody diarrhea was evident in 100% of infected birds followed by soiled vent (82.20%), inappetence (73.50%), ruffled feathers (73.40%), depression (68.20%), dropped wings (65.20%), and drowsiness (52.40%). In supplemented and treated groups (G-I, G-II, G-III, G-IV, and G-V), the symptoms were milder than infected non-treated group (G-VI) as birds showed depression, decreased activity and slight bloody diarrhea on 5th and 7th dpi then returned to the normal condition. Group II and Group III (supplemented with 0.5% and 0.75% guava leaf extracts per liter drinking water, respectively) showed depression, ruffled feathers, bloody diarrhea, and a mortality rate of 7.5%. On the other hand, the infected non-treated group (G-VI) showed ruffled feathers, a decrease in appetite, depression, bloody diarrhea, paralysis, and a mortality rate of 46.3%. Symptoms started from the 4th dpi with maximum strength on the 8th and 9th dpi and then regression and disappearance of bloody diarrhea till the end of the experiment. Bloody diarrhea and other signs were less severe in guava-supplemented groups than positive control group.

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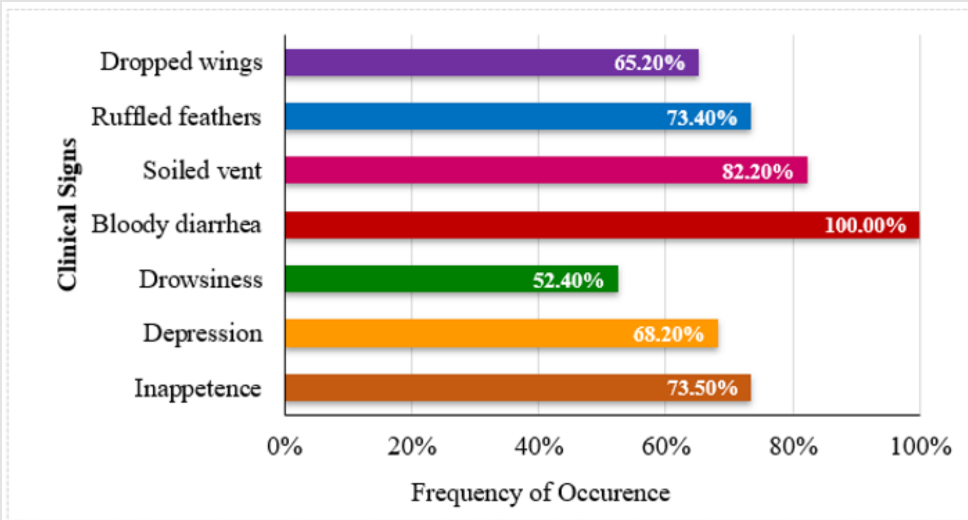

Figure 1: Frequency of clinical signs in experimentally induced coccidiosis in broiler chicken. Each bird was infected orally with 10,000 sporulated oocysts of mixed Eimera species. The oocyst mixture used comprised 30% *E. tenella*, 20% *E. maxima*, 20% *E. acervulina*, 15% *E. necatrix*, and 15% *E. mitis*.

Effects of Aqueous Leaf Extract of *P. Guajava* on Fecal Score and Mortality Rate

Table 1 clearly shows that mortality was higher in the infected, untreated control group (G-VI) compared to that of the treated groups. Among the treated groups, mortality was numerically lowest (5.0%) in the group (G-V) treated with synthetic anticoccidial (Amprolium). In the extract-treated groups, the highest mortality was observed in the group (G-I) treated with 0.25% of *P. guajava* leave extract while the groups treated with 0.5% (G-II) and 0.75% (G-III) of *P. guajava* extracts exhibited similar mortality rates (7.5%). In the extract-treated groups, the lowest mortality (6.3%) was observed in the group (G-IV) treated with 1% of *P. guajava* leave extract. As expected, infected positive control (G-VI) had the highest mortality rate (46.3%) while only 1.3% mortality rate was recorded in non-infected group (G-VII). The fecal scores were determined for all groups of birds by a daily inspection of the droppings from 1st dpi to 10th dpi. A qualitative assessment of the appearance of droppings was done to differentiate abnormal droppings from normal one. Dropping of the G-VII (NC) was considered as standard normal droppings of broiler raised on litter. A fecal score of 0 indicates normal droppings; whereas, a fecal score of III indicates maximum deviation of the feces from normal. Bloody/tan colored diarrhea were observed in all the treatment groups at the onset of treatment as shown in Table 2. The degree of bloody diarrhea in broiler chickens treated with synthetic anticoccidial (G-V) was milder compared with groups treated with aqueous extracts of *P. guajava* leaves. However, the mildness observed in the groups treated with the extract of *P. guajava* varied in a concentration dependent manner (Table 2).

Table 1: Effect of treatments on mortality rate in broilers (n = 80/group) experimentally inoculated with sporulated oocyts of mixed *Eimeria spp*.

Groups (n=80)	Treatment (dose/ lit DW)	Number of Mortality (dpi)							Total Mortality (No.)	Percentage (%) of Mortality
		8th	9th	10th	11th	12th	13th	14th		
G-I	PE 0.25%	6	3	1	3	1	0	0	14	17.5%
G-II	PE 0.5%	4	1	0	0	1	0	0	6	7.5%
G-III	PE 0.75%	2	1	2	1	0	0	0	6	7.5%
G-IV	PE 1.0%	3	0	2	0	0	0	0	5	6.3%
G-V	SA 0.125%	2	1	1	0	0	0	0	4	5.0%
G-VI	PC	8	10	7	5	6	1	0	37	46.3%
G-VII	NC	0	1	0	0	0	0	0	1	1.3%

Note: PE: Plant Extract; SA: Synthetic Anticoccidial; PC: Positive Control (infected, untreated); NC Negative Control (uninfected, untreated).

Table 2: Fecal score of broiler chickens experimentally infected with mixed species of *Eimeria* and treated with leaf extract of *P. guajava* and synthetic anticoccidial Amprolium.

Groups (n=80)	Treatment (dose/lit DW)	Fecal Score (dpi)									
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
G-I	PE 0.25%	-	+	+	+ +	+ +	+ +	+ +	+ + +	+ + +	+ +
G-II	PE 0.5%	-	+	+	+ +	+ +	+ +	+ +	+ + +	+ + +	+ +
G-III	PE 0.75%	-	+	+	+ +	+ +	+ +	+ +	+ + +	+ + +	+ +
G-IV	PE 1.0%	-	+	+	+ +	+ +	+ +	+ +	+ + +	+ + +	+
G-V	SA 0.125%	-	+	+	+	+	+ +	+ +	+ +	+ +	+
G-VI	PC	-	+	+ +	+ + +	+ +	+ +	+ + +	+ + +	+ + +	+ +
G-VII	NC	-	-	-	-	-	-	-	-	-	-

Note: PE: Plant Extract; SA: Synthetic Anticoccidial; PC: Positive Control (infected, untreated); NC Negative Control (uninfected, untreated).

Effects of Aqueous Leaf Extract of *P. Guajava* on Lesion Score

The severity of congestion, hemorrhage, and ballooning of ceca in guava-supplemented groups (G-I, G-II, G-III, G-IV) was less severe compared with synthetic anticoccidial (G-V) and positive control (G-VI) groups (Table 3). Commonly all infected supplemented and treated groups showed a gradual increase of congestion of all intestinal mucosa and the ceca were ballooned from 5th to 9th dpi. On the other hand, G-VI (infected and non-treated) showed severe congestion, an increase in the thickness of the intestinal wall, severe hemorrhage, ballooning of the intestine and the presence of bloody cecal core from 5th to 7th dpi. The severity of congestion gradually decreased within 10 and 11th dip. Furthermore, G-VII (non-infected and non-treated) showed no lesions. The clinicopathological comparisons of normal and experimentally induced coccidiosis in broiler chicken are illustrated in figure 2.

Table 3: Lesion score of broiler chickens experimentally infected with mixed species of *Eimeria* and treated with leaf extract of *P. guajava* and synthetic anticoccidial Amprolium.

Groups (n=80)	Treatment (dose/ lit DW)	Lesion Score (dpi)									
		4th	5th	6th	7th	8th	9th	10th	11th	12th	13th
G-I	PE 0.25%	0	I	II	II	III	III	II	II	I	0
G-II	PE 0.5%	0	I	I	II	III	III	II	II	I	0
G-III	PE 0.75%	0	I	I	I	II	II	II	I	I	0
G-IV	PE 1.0%	0	0	I	I	II	II	II	I	I	0
G-V	SA 0.125%	0	I	II	II	II	III	II	I	I	0
G-VI	PC	0	II	III	III	III	II	II	II	I	0
G-VII	NC	0	0	0	0	0	0	0	0	0	0

Note: PE: Plant Extract; SA: Synthetic Anticoccidial; PC: Positive Control (infected, untreated); NC Negative Control (uninfected, untreated)

Figure 2: Clinicopathological comparison of normal and experimentally induced coccidiosis in broiler chicken.

A. Normal droping of healthy broilers
B. Bloody diarrhea in experimentally induced coccidiosis in broilers.
C. Normal ceca of healthy broiler
D. Cecal filled with bloody intestinal contents in infected birds.
E. Congested and engorged intestines of experimentally infected birds with mixed coccidia.
F. Intestine of healthy broiler (noninfected).
G. Ballooning of intestine of infected broilers.
H. Healthy intestinal mucosa of noninfected broilers.
I. Congested and hemorrhagic intestinal mucosa of infected broilers.

Effects of Aqueous Leaf Extract of *P. Guajava* on Oocysts Count

The final fecal oocyst counts in *Eimeria*-infected broilers treated with extracts in different groups revealed a notable reduction in oocyst count, with the decrease being related to the dosage used in the treatment groups (Table 4). The highest oocysts count in all infected groups was recorded at 6th, 7th, and 8th dpi. There was a significant increase in total oocyst count in G-VI (infected, non-treated group) (81.42 ± 14.25 x 10^3) on 6th dpi compared with other groups. Oocyst counts were remarkably decreased with days of treatments in all supplemented groups as well as group treated with synthetic anticoccidial. Most significant decrease in total oocysts counts were evident from 8th dpi in G-IV (3.32 ± 0.91 x 10^3) and G-V (3.042±0.32 x

10^3) compared with other supplemented groups (G-I, G-II, G-III) and infected, non-treated group (G-VI) (4.82±1.18 x 10^3, 4.1±0.72, x 10^3, 3.93±0.65 x 10^3 and 68.4±15.48 x 10^3), respectively. The highest oocyst reduction rate was 94.6% in the group that received the standard anticoccidial drug. Among the groups treated with plant extracts, the highest reduction rate was 92.7% at a dose of 1% of plant extracts, which decreased in a dose-dependent manner to 91.7%, 90.4% and 90.5% in groups treated with 0.75%, 0.50% and 0.25% of plant extracts per liter of drinking water, respectively. Oocysts reduction rate in positive control (infected but not treated) was 67.7% (Figure 3).

Table 4: The number of oocysts per gram of feces (×10^3) in different treatment groups in relation to days of post infection (dpi).

Groups (n=80)	Treatment (dose/lit DW)	Oocyst count (dpi)					
		5th	6th	7th	8th	9th	10th
G-I	PE 0.25%	10.35±1.22^{a}	8.98±1.4^{a}	6.11±1.73^{a}	4.82±1.18^{a}	2.86±0.96^{a}	0.98±0.32^{a}
G-II	PE 0.5%	8.92±2.01^{b}	7.82±1.14^{b}	5.73±0.93^{b}	4.1±0.72^{a}	2.41±0.81^{a}	0.86±0.38^{a}
G-III	PE 0.75%	8.86±1.43^{b}	6.24±0.91^{b}	5.01±1.01^{b}	3.93±0.65^{b}	2.1±0.62^{a}	0.74±0.41^{a}
G-IV	PE 1.0%	8.36±0.97^{b}	5.18±1.4^{c}	4.46±0.89^{b}	3.32±0.91^{b}	1.82±0.32^{a}	0.61±0.22^{a}
G-V	SA 0.125%	7.74±0.61^{c}	4.61±0.71^{c}	3.92±0.64^{c}	3.04±0.32^{b}	0.8±0.41^{b}	0.42±0.19^{a}
G-VI	PC	72.51±12.5^{d}	81.42±14.25^{d}	74.14±12.68^{d}	68.4±15.48^{d}	61.21±11.72^{c}	23.4±7.6^{b}
G-VII	NC	0^{e}	0^{e}	0^{e}	0^{e}	0^{d}	0^{e}

Note: Values are presented as Mean ± SEM. Values carrying different superscripts in columns differ significantly (p<0.5). PE: Plant Extract; SA: Synthetic Anticoccidial; PC: Positive Control (infected, untreated); NC Negative Control (uninfected, untreated).

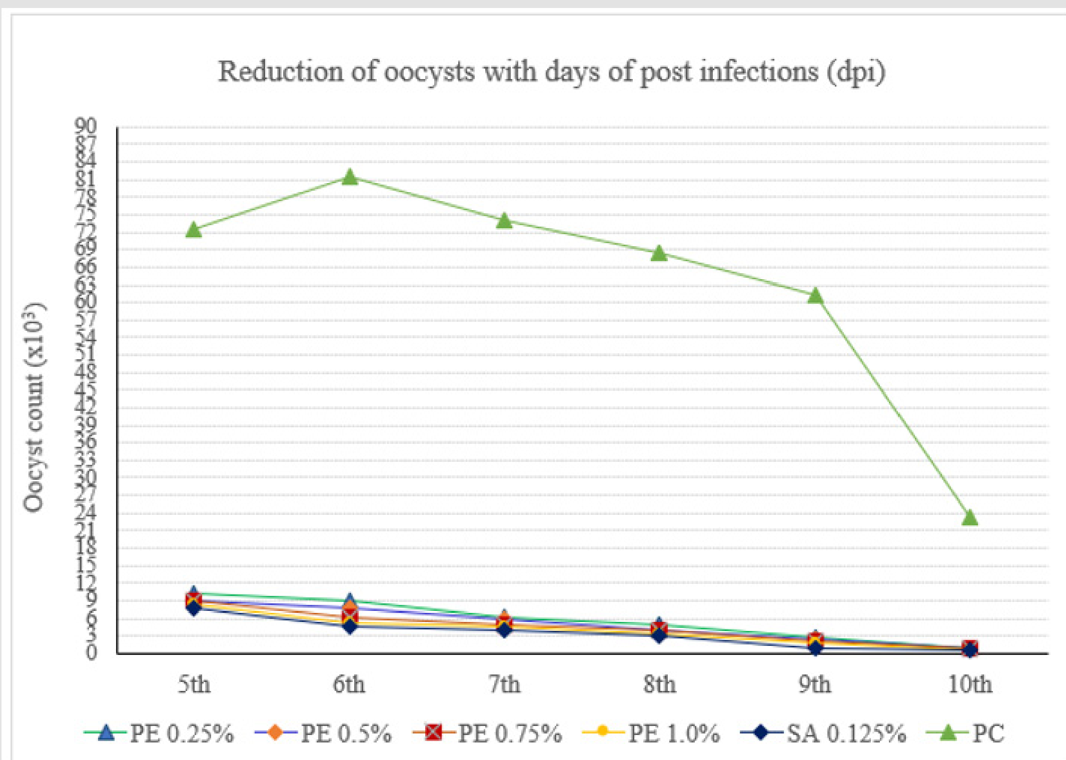

Figure 3: Effects of herbal extracts on fecal oocyst counts and their reduction in Eimeria-infected broilers in different treatment groups in comparison to positive control (infected but untreated). PE: Plant Extract; SA: Synthetic Anticoccidial; PC: Positive Control (infected, untreated); NC Negative Control (uninfected, untreated).

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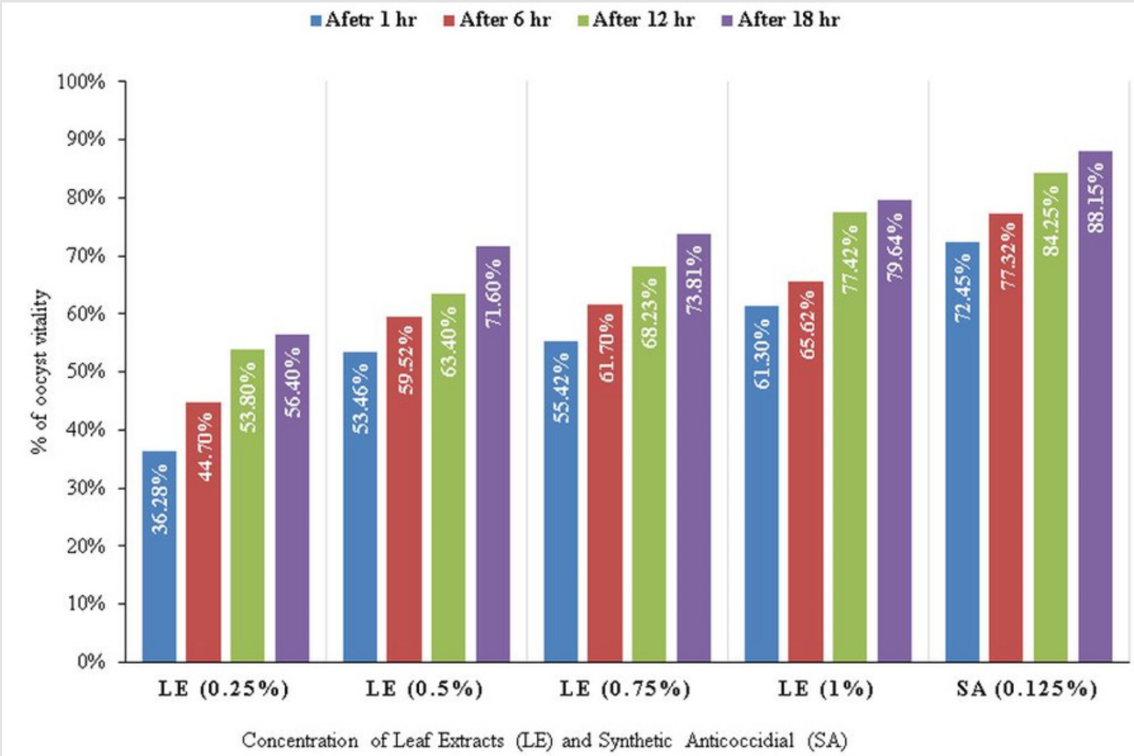

Figure 4: *In vitro* efficacy of Guava leaf extracts (LE) and Synthetic Anticoccidial (SA) on oocyst vitality.

Effects of Aqueous Leaf Extract of *P. Guajava* on Oocyst Vitality

Different concentrations of *P. guajava* extracts showed concentration-dependent inhibition for the viability of coccidial oocysts of different *Eimeria* species as compared to the control group as shown in Figure 4. According to current study results, aqueous extracts exhibited good inhibition for the viability of coccidial oocysts against *E. tenella, E. maxima, E. acervulina, E. neatcrix* and *E. mitis*. The highest efficacy guava leaf extract was 79.64% at the concentration of 1g/liter distilled water after 18hrs of exposure. The lowest efficacy was 56.40% at the concentration of 2.5g/liter distilled water after 18hrs of exposure. However, synthetic anticoccidial Amprolium showed the highest efficacy (88.15%) against oocyst vitality in experimental condition. Nevertheless, the efficacy of guava leaf extract was nearly similar to that of Amprolium.

Discussion

Coccidiosis is a globally occurring economic health hazard of chicken that can infect any type of poultry in any type of facility [30]. The severity of *Eimeria* infection in chickens is usually assessed by loss of body weight gain, excretion of fecal oocysts, and the presence of intestinal lesions [4]. The present study was planned to evaluate an *in- vitro* efficacy of different concentrations of guava leaf extracts on mixed *Eimeria* oocysts vitality and application of the most effective concentrations *in vivo* on the experimentally infected broiler chicks with local isolates of mixed *Eimeria* species. The present work showed the detrimental effects of coccidiosis on clinical signs, mortality rate, lesion scoring, and oocysts count after challenging the chicks with oocysts of mixed *Eimeria* sp. These findings demonstrated the successful experimental induction of coccidiosis in broilers with field isolates that confirmed the pathogenicity of Bangladeshi field isolates. The *in vitro* study revealed that the aqueous extracts of guava leave induced anticoccidial effect which was concentration dependent and increased by increasing the concentration of the tested extracts. Guava leaves by its high concentration exerted the greatest percentage reduction in oocysts count and vitality and had high *in vitro* anticoccidial effect (Table 4, Figure 2).

Although most of the studies conducted elsewhere were mainly on the antibacterial activities of *Psidium guajava*, present study findings are in good agreement with other studies conducted elsewhere on herbal remedies of coccidiosis in chicken [21,31]. While a different botanical remedy for avian coccidiosis namely pine bark (*Pinus radiata*) was studied, Molan et al 2009 [32] also reported similar *in vitro* sporulation inhibition activities of aqueous extracts of the plant against three species of avian coccidia which were also in agreement with present study findings indicating that botanical could be a potential alternative to synthetic anticoccidial drugs [13,27]. *P. guajava* extracts have been reported to inhibit the endogenous enzyme activities [33]. Therefore, it might be that *P. guajava* extract reduced the proportion of oocysts sporulation by inhibiting or inactivating the enzymes responsible for the sporulation process as in helminth eggs [32]. Likewise, Jones et al. 1994 suggested that extracts may infiltrate the cell wall of oocysts and cause a loss of intracellular components [34,35]. However, the mechanism of inhibition is unknown, but it might be linked to osmotic effects attributed to extracts. Extracellular calcium and Ca+ signaling are essential for the invasion of *Eimeria* into host cells [35]. Extracts have been shown to trigger and desensitize receptors in calcium channels [36].

It is likely that *P. guajava* leaf extracts contributed to the observed inhibition of oocysts viability by disrupting calcium-mediated signaling. High mortality rate was recorded in the infected non treated group primarily during the first days after inoculation (Table 1). The detrimental effect of infection was also reflected in the appearance of bloody diarrhea during 4-6 days after infection (Figure 1) while the most severe lesion scores of the duodenum and cecum were recorded at 5^{th}, 6^{th}, 7^{th} and 8^{th} dpi (Table 3). The highest total oocysts count recorded in the droppings collected on 5^{th}, 6^{th}, 7^{th} ,8^{th} and 9^{th} dpi (Table 4). The tested herbal extracts were shown to alleviate the lethal effects of the experimental infection in chicken as the oocysts count and lesion scores were reduced with days of treatment. The best results were obtained in groups treated with synthetic anticoccidial Amprolium and 1% guava leaf extract/lit drinking water as compared with other groups including infected non treated group. The mortality rate was reduced in a dose dependent manner in all supplemented groups as compared with infected non treated group. However, the groups treated with 0.5% (G-II) and 0.75% (G-III) of *P. guajava* extracts exhibited similar mortality rates (7.5%).

Only 1.3% mortality was recorded in Group VII (Non-infected – non-treated) which is in usual range for broiler production. The results of the present study are in agreement with Elmorsy et al 2016 who observed the highest mortality in infected non-medicated control group in Japanese quail [37]. The present findings are also supported by Ahmed, et al 2018 who studied *in vivo* anticoccidial activity of crude leaf extracts of *Psidium guajava* in broiler chickens [38]. Bloody diarrhea was evident in all the experimental groups, except the uninfected control group, but the extent of bloody diarrhea in the groups supplemented with Amprolium and the tested extracts of guava leaves at concentrations of 0.75% and 1.0% were milder than that of other groups. The oocysts count and lesion score were zero in uninfected groups. Large number of oocysts was produced in all the infected groups. However, addition of either of the tested plant extracts and synthetic anticoccidial to drinking water of infected chickens significantly decreased the total oocysts count and improve lesion score indicating the potentials of guava leaf extracts in reducing pathogenicity of *Eimeria* species in broilers.

The efficacy of the test material in reducing the fecal oocysts density of *Eimeria spp*. is interesting. This desirable effect could be attributed to the phytochemical contents of the plant. Anyanwu and Dawet 2005, revealed that plant chemicals like those found in the leaf of the test plant are effective against protozoa of which *E. tenella* is an example [39]. One of the phytochemicals, saponin, earlier reported to be present in the leave of the plant [40] has been found to bind with the sterol molecules on the cell membrane of parasites and destroy them [41,42]. The effect of the screened plant in reducing the fecal oocyst density is also similar to its activity against other related organisms such as *G. lamblia* [31], *T. gondii* [43], *T. brucei brucei* [44] and *Plasmodium spp* [45]. This highlights its usefulness in the management of protozoan parasitic infection. The alleviation of cecal lesions exhibited by the treatment plant is impressive. This might be due to its ability to depress oocyst multiplication. It might also be associated with the improvement of the harmful effects of the protozoa probably through its antioxidant as well as free radical scavenging properties as observed by earlier workers [46,47]. The observation of attenuation of the cecal lesion in this study is similar to the findings of Lee at al 2013 who revealed that *P. guajava* leave effectively reduced lesions by another coccidian, *Toxoplasma* [44].

P. guajava is known for their antioxidant properties [48]. It might be that antioxidant-rich plants are lethal to the parasites by inducing oxidative stress and neutralize reactive oxygen species and have potential benefits in treating coccidial infections. Antioxidant compounds are known to reduce the severity of *Eimeria tenella* infections by ameliorating the degree of intestinal lipid peroxidation. Plants of the genus *Psidium*, including *P. guajava* (Guava), has antioxidant and antiinflammatory properties which could be detrimental for disease outcome [43]. Moreover, the lower oocysts count recorded in the infected groups given the herbal extracts was probably due to the presence of the phenolic compounds in the tested herbal powders [40]. Phenols can interact with cytoplasmic membranes and change their cation permeability, leading to impairment of crucial processes in the coccidia cells and, finally, their death [49]. The reduction of the cecal damage and the partial inhibition of the *Eimeria spp.* oocyst multiplication identified in this study as well as the earlier reported antibacterial activities of the leaves of the test plant appeared to be responsible for the decreased severity of diarrhea observed in the treated birds. One of the complications of coccidioisis which contributes to the development of clinical signs and intestinal pathology is secondary infection by enteric bacteria such as *Escherichia coli, Salmonella spp* etc. [50].

The leaf of the test plant has been shown to inhibit these bacteria [40,51-53]. Thus, complication arising from secondary infections are eliminated which reduced the bloody feces. Furthermore, the effectiveness of the test material in minimizing blood loss is worth noting. This verifies the report of [54] who showed that guava leaves contain B complex vitamins, Niacin, Folate, Iron, Zinc and Copper all of which contribute to blood formation. Therefore, consumption of the test plant will have an additional advantage of being a food supplement that will enhance blood production and resistivity to infection aside from its role in managing coccidiosis. Reduction in morbidity and mortality of the affected birds is undoubtedly advantageous to the farmer since the threat of losing an entire flock is substantially eliminated. Our result is also in consonance with the report of Sahoo et al., 2016 that guava leaves contain Vitamin C which is known to have immunostimulatory effects leading to resistivity to disease [55,56].

Conclusion

The present study confirms that *P. guajava* possesses anticoccidial property. The study findings clearly demonstrated that guava leaf extracts exhibited anticoccidial activity which was comparable with that of the synthetic anticoccidial drug. The results of the present study suggest that supplementation with guava leaf extracts at concentrations of 0.75% and 1% per liter drinking water significantly alleviates the negative impact of *Eimeria* infection in broiler chickens. The anticoccidial properties of guava leaf extracts were evidenced by the prevention or reduction of mortality, lowering gut lesion scores and reduced fecal oocyst shedding of the infected chickens as compared to the infected non treated group. It can be concluded from our data that guava leaf extracts exhibit a significant anticoccidial activity and can be recommended for the treatment and control of avian coccidiosis. However, further advanced studies should be conducted to investigate the mechanism of action and potential therapeutic use of this plant against coccidiosis in chickens.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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