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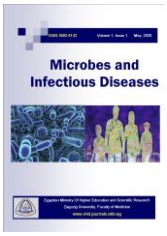

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Original article

Assessment of the synergistic effects of *Hibiscus sabdariffa* calyx extract and anticoccidial drugs against *Eimeria* infections in broiler chicks

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ABSTRACT

Background: Coccidiosis, caused by *Eimeria* species, is a major disease affecting poultry industry causing economical loses. Anticoccidial drugs are commonly used to control coccidiosis; however, drug resistance and residual effects in poultry products necessitate alternative strategies. This study investigates the potential synergy of aqueous extract of *Hibiscus sabdariffa* calyces (AEHS) with selected anticoccidial drugs in the treatment of Eimeria-infected broiler chicks. **Methods**: Forty broiler chicks were obtained, raised for three weeks and divided into eight groups. Faecal samples were collected, and the birds were inoculated with *Eimeria* oocysts. Treatment regimens were administered for five days. Clinical signs were monitored, and faecal samples were analyzed for oocysts, and bacteria counts. **Results**: AEHS exhibited significant synergistic effects with all tested anticoccidial agents, with the most notable impact observed with diclazuril, resulting in the complete clearance of the initially high oocyst per gram (OPG) (2058.5±1314.51). Body condition improved in all medicated groups post-treatment, with a significant reduction in symptoms ($p< 0.05$). In terms of weight gain, significant differences ($p< 0.05$) were observed between treated groups and the untreated control. The highest weight gain was in the diclazuril with AEHS group, while the lowest was in the amprolium-only group. AEHS alone demonstrated moderate weight gain, suggesting its potential as a standalone anticoccidial treatment. Bacterial counts were also reduced effectively by AEHS in combination with anticoccidial drugs. **Conclusion**: This study demonstrated the potential synergistic effects of AEHS in mitigating *Eimeria* infections in broiler chicks. Further research is needed to explore underlying mechanisms and optimize the treatment regimens.

Introduction

Eimeria infections, caused by a group of protozoan parasites, significantly impact the poultry industry, leading to reduced growth rates, increased mortality, and economic losses. Coccidiosis resulting from the pathogenic *Eimeria* species is usually characterized by dysentery, enteritis, diarrhea, which may be bloody and sometimes, emaciation, lower feed conversion rate, delayed sexual maturity, drooping wings, poor growth and

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low production with attendant high mortality and morbidity rates [1,2]. About nine species of *Eimeria* have been recognized in domesticated chickens out of which *Eimeria brunette*, *Eimeria maxima*, *Eimeria necatrix*, and *Eimeria tenella* are the most pathogenic. *Eimeria acervulina*, *Eimeria imitis*, *Eimeria mivati* are the less pathogenic and *Eimeria praecox* and *Eimeria hagani* have mild pathogenicity [2].

Anticoccidial drugs are commonly used to control coccidiosis; however, emerging drug resistance and concerns over residual effects in poultry products necessitate alternative strategies. One potential solution to the aforementioned menace is the use of natural plant extracts, such as *Hibiscus sabdariffa* calyx extract, which has shown promising antimicrobial, antioxidant properties and antihypertensive properties in animal and human models [3,4]. The plant has also been reported to have antibacterial and antiparasitic activities but anticoccidial properties against *Eimeria* infections has not been explored. Hence, this study aimed at evaluating the potential synergistic effects of AEHS with selected anticoccidial drugs in mitigating *Eimeria* infections in broiler chicks.

Materials and methods

Ethics

All activities in this research were one following all research and animal handling ethics and the research was approved by Kwara State University ethical committee before the commencement of the study with approval number KWER/21/57MMB/003.

Source of *Eimeria* oocyst

Eimeria oocyst used for this study was obtained from the Department of Veterinary Parasitology and Entomology, University of Ilorin, Nigeria.

Sources of drugs

The drugs used for the study were obtained from commercial Veterinary store (Animal Hope Veterinary Solutions) in Ilorin.

- Amprolium 20 % KEPRO B. V, Maagdenburgstraat 17-7421 ZA Deventer-Holland.
- Diclazuril 2.5% Aetter centre (Beijing) Biology. co., Ltd.
- Totralzuril2.5% Aetter centre (Beijing) Biology. co., Ltd.
- Sulphaqunoxalline Sulphadimidine, KEPRO B. V, Maagdenburgstraat 17-7421 ZA Deventer-Holland.

The chemicals were reconstituted according to manufacturers' recommendation.

Plant authentication and preparation

Dried calyces of *Hibiscus sabdariffa* were obtained from Oja Oba in Ilorin, Kwara State, Nigeria. The calyx was taxonomically identified and authenticated in the herbarium of the Department of Plant Biology, University of Ilorin with voucher number UIL-V-1084. The calyces were thoroughly washed and dried at room temperature. The dried calyces were then grinded to powder using pestle and mortar (Cole-Parmer®). About 2 kg of powdered calyces was mixed with 5 L of distilled water to obtain 40 % (w/v) and this was allowed to stand for 72 hours on the laboratory bench at 25 ^{0}C. It was filtered and the filtrate was concentrated to dryness in water bath (Lab Fish®) to produce the residue called extract. The extract was further air dried on the laboratory shelf. The extract was labelled aqueous extract of *Hibiscus sabdariffa* (AEHS) and stored in a refrigerator.

Experimental animals and study designs

Forty broiler chicks were purchased from Agrited hatchery in Ibadan, Oyo State, Nigeria. The chicks were brooded for 10 days under stable environmental conditions with only vitamins (Stressroak®). The management system was intensive deep litter system under strict biosecurity measures. The birds were fed with standard crumble feeds (Chikun® Ilorin, Nigeria) and clean medication free water provided *ad libitum*. No anticoccidial drugs were given at this period. At the third week, the birds were divided into eight groups (A-E synergy test group, F-control, G and H anticoccidial-only test group) of 5 birds each the study using the case control approach. The dosages of the drug and extract used in the experiment were as presented in Table 1. The birds in each group were weighed and they were observed for any coccidiosis symptoms. Thereafter, faecal samples were collected from the chicks in each group and samples were screened for coccidiosis using floatation techniques method as previously described [5].

Eimeria stock and inoculation

The field strains of *Eimeria* oocyst were identified to contain *Eimeria tenella* and *Eimeria necatrix* on the basis of morphology using

morphological keys as described by **Taylor *et al.*** [6]. The chicks that were not shedding oocysts were infected with sporulated Eimeria oocysts at 3 x 10^3 OPG. Fecal samples were collected from the chicks 72 hours after experimental infection and were subsequently dispatched to the Microbiology laboratories at Kwara State University for the quantification of *Eimeria* and associated secondary bacterial counts.

Treatment of infected chicks

The birds were re-weighed and clinical signs observed. Following establishment of infection with clinical signs and faecal shedding of oocyst, treatment began in each group for 5 days via oral route.

Sample collection

After five days of treatment, the chicks were observed for clinical signs of coccidiosis and faecal samples were collected using sterile swab stick (Puritan) via cloaca swabbing. Freshly voided faeces were also collected from each group into sterile sample bottles. The swabs and faecal samples were transported within one hour of collection to Microbiology laboratory, KWASU under cold chain for Microbiological and Parasitological analyses respectively. In addition, the weights of the chicks were taken and recorded. The treatment regimens for each group are clearly outlined in **table (1).** Each group is given a different treatment regimen for 5 days. The different treatments include various combinations of AEHS, anticoccidial drugs, and water. The duration of treatment is the same for all groups (5 days).

Mesophilic viable bacterial counts and Coliform counts

The faecal sample swab stick was inoculated in 10 ml of buffered peptone water and incubated at 37 ^{0}C for 24 hours and this was used as stock. One milliliter of stock sample was dispensed into a sterile tube with 1 ml of normal saline and homogenized for even distribution. The tube was allowed to stand for 5 minutes, followed by 10-fold dilution of 0.5 ml of the supernatant in tubes containing 4.5 ml of normal saline. MacConkey and Nutrient agar were used for coliform and total viable bacteria count respectively using Miles-Mistral technique of bacteria count [7]. Each agar was divided into eight sectors and each sector were inoculated with 20 µl of appropriate dilution factors (10^{-10}) (avoiding the tips from touching the agar surface) and were allowed to dry (within 20 minutes). Subsequently, the plates were inverted and incubated at 37 ^{0}C for 24 hours. Control plates were also made by inoculating agar with 20 µl of normal saline. Discrete colonies in each sector were counted visually and the estimated viable and coliform count were calculated using the formular

Colony forming unit (CFU)/ml/g of **f**aece = Average number of colonies for a dilution X dilution factor.

Oocyst count in faeces

Four grams of feces were weighed and transferred into a container, with a sugar solution serving as the flotation medium, following the method described by **Soulsby** [5]. The contents in the container were thoroughly mixed using a spatula. Subsequently, the fecal suspension was filtered through a double layer of cheesecloth into a second container. This filtrate was further agitated in the second container with a Pasteur pipette.

A sub-sample was then withdrawn using a pipette while the filtrate was being stirred. The fluid was mixed, and the first compartment of the MacMaster counting chamber was filled with this sub-sample. After stirring the fluid once more, the second compartment of the MacMaster counting chamber was also filled with another sub-sample. The counting chamber was left undisturbed for 5 minutes, allowing the eggs to rise to the surface and debris to settle at the chamber's bottom.

Subsequently, the sub-sample of the filtrate was examined under a compound microscope at a magnification of X100. All eggs within the marked area were identified and enumerated.

The number of eggs per gram was calculated by:

1. Counting the number of eggs within the grid of each chamber, ignoring those outside the squares.
2. The total was then multiplied by 50.

Statistical analysis

The data obtained were entered into Statistical package for social sciences (SPSS) for descriptive analysis. Mean and standard deviation were obtained and results were presented in standard form.

Results

The AEHS show synergistic effect on all the anticoccidial agents tested in this study with the most effect exhibited by combination AEHS and diclazuril which totally cleared the initially high oocyst per gram (2058.5 ± 1314.51) of birds in

group B. Generally, the oocyst per gram of faeces was higher before treatment in all the medicated groups than after treatment ($p< 0.05$). Although, the oocyst shedding after treatment with AEHS extract is not lower than initial OPG before treatment in group E, the increase in OPG is not statistically significant when compared with other treated groups ($p>0.05$) but statistically significant ($p<0.05$) when compared with infected but not treated control group **(Table 2).**

The combination of AEHS with conventional anticoccidial drugs showed a synergistic effect in reducing the oocyst count in Eimeria-infected broiler chicks. AEHS alone also showed some efficacy in reducing the oocyst count, but the effect was not as significant as when used in combination with conventional drug.

The classical clinical signs of coccidiosis including bloody diarrhoea, lethargy, drooping wing, ruffle feather and death were noted among the chicks in all groups following infections. The clinical signs recessed in all the medicated groups but became aggravated in untreated control group **(Table 3). Table 3** shows the clinical signs of coccidiosis in chicks with acute Eimeria tenella infection before and after treatment with AEHS alone and in combination with selected conventional anticoccidials. Before treatment, all medicated groups showed clinical signs of coccidiosis, including bloody diarrhea (BD), decreased water intake (DW), ruffled feathers (RF), lethargy (L), and death (D). After treatment, the clinical signs improved in all medicated groups, with a significant decrease in BD, DW, and RF ($p<0.05$). The combination of AEHS and diclazuril showed the highest improvement in clinical signs, with no deaths observed after treatment. Group E, which received only AEHS extract, also showed improvement in clinical signs, with no deaths observed after treatment. Group H, which was treated with only diclazuril, showed improvement in clinical signs, but one bird died after treatment.

The AEHS extract showed a synergistic effect with the conventional anticoccidials used in the study, improving the clinical signs of coccidiosis in broiler chicks. The combination of AEHS and diclazuril was the most effective treatment, with no deaths observed after treatment. AEHS alone also showed potential as an alternative treatment for coccidiosis in broiler chicks.

The average weight gain by chicks after treatment down the all groups was significantly difference ($p< 0.05$) from that of untreated control **(Figure 1). Figure 1** shows the weight gain of chicks infected with *Eimeria tenella* and treated with AEHS (aqueous extract of *Hibiscus sabdariffa* calyces) alone and in combination with selected conventional anticoccidial drugs. The weight gain was highest in group A (diclazuril with AEHS) with a value of 1,050 g. The weight gain was lowest in group G (amprolium only) with a value of 750 g. The weight gain in group E (AEHS only) was 900 g. The weight gain in groups B, C, D, and H ranged from 850 g to 950 g. The combination of AEHS with diclazuril (group A) resulted in the highest weight gain, indicating a potential synergistic effect between the two treatments. The use of AEHS alone (group E) resulted in a moderate weight gain, suggesting that AEHS may have some anticoccidial activity on its own. The use of conventional anticoccidial drugs alone (groups G and H) resulted in the lowest weight gain, indicating that these drugs may not be as effective as the combination treatments. **Table 4** shows the bacteria count of chicks infected with *Eimeria tenella* before and after treatment with AEHS (aqueous extract of Hibiscus sabdariffa calyces) in combination with selected conventional anticoccidial drugs. The groups are labeled A to H, with each group receiving a different treatment regimen. The bacteria count is measured in terms of total bacterial count (TBC) and coliform counts. Before treatment, the TBC and coliform count were highest in group H, which was treated with only diclazuril. After treatment, the TBC and coliform counts were lowest in group C, which was treated with amprolium and AEHS extract. The combination of AEHS extract with conventional anticoccidial drugs generally resulted in a decrease in TBC and coliform counts after treatment. The use of AEHS extract in combination with conventional anticoccidial drugs can effectively reduce bacterial counts in chicks infected with *Eimeria tenella*. The combination of amprolium and AEHS extract was the most effective in reducing bacterial counts.

Table 1. Treatment regimen for the chicks in each group.

Group	Treatment	Duration
A	AEHS at 10g in 4litres and *totralzuril at 1ml in 1litre	5 days
B	AEHS at 10g in 4litres and *diclazuril at 1ml in 4litres	5 days
C	AEHS at 10g in 4litres and Amprolium at 1gm in 2litres	5 days
D	Hibiscus Sabdariffa at 10gm in 4litres and sulphadimidine at 1gm in 2 litres	5 days
E	Hibiscus Sabdariffa at 10gm in 4litres	5 days
F	Water ad libthum and not infected	5 days
G	Amprolium at 1gm in 2litres	5 days
H	Diclazuril at 1ml in 4litres	5 days

AEHS: Aqeous extract of *Hibiscus sabdariffa* *Totrazul and diclazuril stopped at day 2

Table 2. Oocyst counts of chicks infected with *Eimeria tenella* treated with AEHS in combination with selected conventional anticoccidial.

Group	Before treatment	After treatment
	OPG ($x10^{10}$)	OPG ($x10^{10}$)
A	534 ± 55.87	5.67 ± 9.81[a]
B	2058.5 ± 1314.51	No egg found [a]
C	70.67 ± 43.66	5.67 ± 9.81 [a]
D	71.00 ± 52.33	No egg found [a]
E	91.67 ± 101.11	104.00 ± 84.25 [a]
F	24.00 ± 10.07	200.67 ± 14.81
G	489 ± 48.90	201.01 ± 10.87 [a]
H	543 ± 43.86	50.47 ± 19.62 [a]

[a]= $P< 0.05$ OPG= Oocyst per gram of faeces

Table 3. The clinical signs of coccidiosis in chicks with acute *Eimeria tenella* infection following treatment with AEHS alone and in combination with selected conventional anticoccidials.

Group	Before Infection					After Inf./Before Rx					After treatment				
	BD	DW	RF	L	D	BD	DW	RF	L	D	BD	DW	RF	L	D
A	0/5	0/5	0/5	0/5	0/5	4/5	3/5	3/5	5/5	2/5	0/3	0/3	0/3	0/3	0/3
B	0/5	0/5	0/5	0/5	0/5	3/5	3/5	3/5	4/5	1/5	0/4	0/4	0/4	0/4	0/4
C	0/5	0/5	0/5	0/5	0/5	5/5	4/5	4/5	5/5	2/5	0/3	0/3	0/3	0/3	0/3
D	0/5	0/5	0/5	0/5	0/5	4/5	3/5	3/5	5/5	1/5	0/4	0/4	0/4	1/4	0/4
E	0/5	0/5	0/5	0/5	0/5	5/5	4/5	4/5	5/5	1/5	1/4	¼	1/4	1/4	1/4
F	0/5	0/5	0/5	0/5	0/5	4/5	4/5	4/5	4/5	2/5	3/3	3/3	3/3	3/3	3/3
G	0/5	0/5	0/5	0/5	0/5	3/5	3/5	3/5	5/5	1/5	2/4	2/4	2/4	2/4	1/4
H	0/5	0/5	0/5	0/5	0/5	4/5	4/5	4/5	5/5	0/5	2/5	2/5	3/5	2/5	1/4

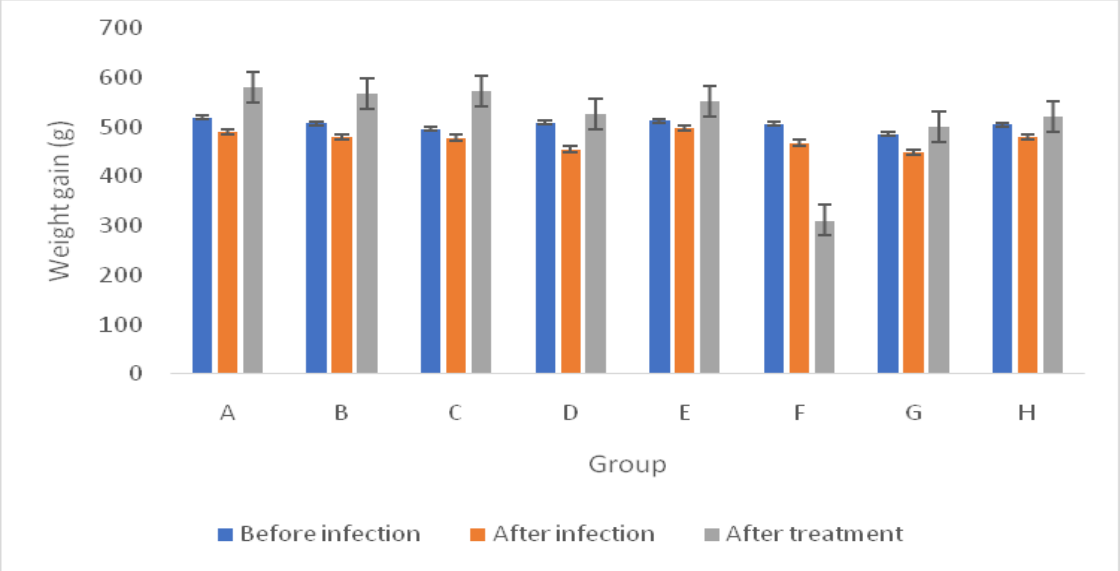
BD= Bloody/Watery diarrhoea, DW= Drooping wing, RF=ruffle feather, L= lethargy, D= Death

Table 4. Bacteria count of chicks infected with *Eimeria tenella* treated with AEHS in combination with selected conventional anticoccidial.

Group	Before Treatment		After Treatment	
	TBC x 10^{10}	Coliform x 10^{10}	TBC x 10^{10}	Coliform x 10^{10}
A	2.03 ± 1.71	0.96 ± 0.77	1.36 ± 1.01*	0.32 ± 1.12*
B	3.20 ± 1.74	1.49 ± 1.60	2.33 ± 1.04 *	1.08 ± 0.46 *
C	2.19 ± 1.68	0.91 ± 0.00	1.55 ± 0.49 *	0.41 ± 0.13 *
D	2.31 ± 1.70	1.34 ± 1.02	1.50 ± 0.87 *	1.05 ± 0.87 *
E	3.00 ± 0.61	1.49 ± 1.60	1.92 ± 1.03 *	1.28 ± 1.03 *
F	1.83 ± 0.31	2.80 ± 0.70	3.75 ± 0.64	4.38 ± 0.40
G	3.16 ± 1.84	2.42 ± 0.93	2.95 ± 1.01 *	1.91 ± 0.23 *
H	3.40 ± 1.76	2.64 ± 1.90	2.85 ± 1.44 *	1.78 ± 1.26 *

* $p< 0.05$ TBC= total bacteria count

Figure 1. The Weight gain of chicks infected with *Eimeria tenella* and treated with AEHS alone and in combination with selected conventional anticoccidial drugs.



Discussion

To the best of our knowledge, this is the first published report of potential anticoccidial activities of AEHS. The findings of the study revealed a significant synergistic effect of the AEHS when combined with selected conventional anticoccidial agents in the treatment of *Eimeria* infections in broiler chicks. Notably, the most pronounced effect was observed in the combination of AEHS with diclazuril, which effectively cleared the initially high oocyst count per gram (OPG) observed in Group B. This observed phenomenon can potentially be attributed to the broader spectrum of activity exhibited by diclazuril against various developmental stages of the parasite, encompassing both the first and second generation meronts, as well as gamont stages [2,8]. Additionally, it is possible that the relatively recent introduction of diclazuril in the Nigerian poultry industry has contributed to its lower resistance levels compared to other established drugs that are long in the market. Studies have shown that diclazuril is effective in reducing oocyst shedding in broiler chickens [9]. In addition, diclazuril is a highly efficacious anticoccidial drug for improving performance and gut health in broilers. In contrast, when considering toltrazuril, a similar anticoccidial drug to diclazuril, administered in combination with AEHS extract to the chicks in Group A, it did not entirely prevent oocyst excretion, even though the initial oocyst burden was lower than that of the chicks in Group B. This occurrence might be associated with the growing resistance to toltrazuril, likely attributed to its frequent and unauthorized use among poultry farmers in Nigeria [10,11]. These results suggest that AEHS, when used in conjunction with traditional anticoccidial drugs, enhances their efficacy in reducing oocyst shedding, similar to the work of **Yang *et al.*** [12] who hypothesized that the combined use of anticoccidial herbs and probiotic in the feed may benefit the control of chicken *Eimeria* infection. The use of several botanical anticoccidial agents such as herbal extracts, aloe vera leaf' aqueous extract and probiotics have been proposed as anticoccidial [13,14].

Furthermore, it was evident that OPG levels were consistently higher before treatment in all medicated groups compared to after treatment ($p < 0.05$). Albeit, the oocyst shedding after treatment with AEHS alone did not lower than the initial OPG before treatment in Group E, the value was not statistically significant when compared with other treated groups ($p > 0.05$). However, it did demonstrate statistical significance ($p < 0.05$) when compared with the infected but untreated control group. The combination of AEHS with conventional anticoccidial drugs displayed a clear synergistic effect in reducing the oocyst count in *Eimeria*-infected broiler chicks. It is worth noting that AEHS, when administered alone, it also exhibited some efficacy in reducing the oocyst counts, although the

effect was not as substantial as when it was used in combination with conventional drugs.

Clinical signs of coccidiosis, including symptoms like bloody diarrhea, decreased water intake, ruffled feathers, lethargy, and death [15] were observed in all groups following the infection. However, there was improvement in all medicated groups after treatment, with a significant reduction in symptoms such as bloody diarrhea, decreased water intake, and ruffled feathers ($p< 0.05$). Notably, the combination of AEHS and diclazuril showed the most significant improvement in clinical signs, with no deaths reported after treatment. Group E, which received only AEHS extract, also demonstrated improved clinical signs, with no post-treatment death. In contrast, Group H, treated solely with diclazuril, experienced an improvement in clinical signs, but one bird succumbed after treatment. However, it is possible that the bird had a pre-existing condition or was infected with a different strain of *Eimeria* that was not susceptible to diclazuril [16].

Additionally, the results indicate that the average weight gains in the treated groups was significantly high ($p < 0.05$) than those of the untreated control group. Similar to other findings, the combination of AEHS with diclazuril (Group A) resulted in the most substantial weight gain, underscoring a potential synergistic effect between these treatments. AEHS used alone (Group E) yielded a moderate weight gain, indicating that AEHS may possess some inherent anticoccidial activity. Conversely, the use of conventional anticoccidial drugs alone (Groups G and H) resulted in the lowest weight gain, suggesting that these drugs may be less effective compared to combination treatments.

Secondary bacterial infection serves as a significant predisposing factor exacerbating the effects and treatment outcomes in coccidiosis [17]. Coccidiosis is a disease that can cause a high bacterial load in the intestine of infected birds. The bacterial load of all the infected groups became high following the manifestation of signs of coccidiosis in all the groups and this is in concordance with previous **Lu *et al.*** [18]. The reduction in bacterial load was noted after the treatment in all the medicated groups. Anticoccidial treatment can shorten the length of illness, decrease discharge of oocysts, alleviate clinical signs, and reduce the likelihood of secondary infections and death [19]. The reduction in bacterial load after treatment may be due to the reduction in the number of oocysts shed by the infected birds and this reaffirms previously reported antibacterial activities of AEHS extract. It was reported that at low concentration, AEHS extract has bacteriostatic effects against wide range of bacteria but it has bactericidal effect at higher concentration [20]. The results revealed that the combination of AEHS with conventional anticoccidial drugs generally led to a reduction in total bacterial counts (TBC) and coliform counts after treatment. Specifically, the use of AEHS extract in combination with conventional anticoccidial drugs proved effective in reducing bacterial counts in *Eimeria*-infected chicks. Coccidiosis is a debilitating disease that cause serious weight loss in the affected animals [21,22]. There was serious weight loss in all the infected group and the mean weight gain generally improves in all the medicated groups including the group with only AEHS extract. This indicate that AEHS also has potential weight gain effect as conventional anticoccidial drugs [10,11,21].

Conclusion

The results of this study indicate that the combination of AEHS with conventional anticoccidial drugs demonstrated a synergistic effect in reducing oocyst counts, alleviating clinical signs of coccidiosis, and promoting weight gain in *Eimeria*-infected broiler chicks. Additionally, AEHS alone showed promising effect as an alternative treatment for coccidiosis. These findings underscore the potential utility of AEHS as a valuable component in the management of coccidiosis in poultry. Further research is warranted to elucidate the mechanisms underlying these synergistic effects and to optimize dosage regimens for maximum effects. The limitation of this study is the inability to fractionate the extract to know the active components and probably synthesize it in the laboratory.

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Authorship

A.A.M, S.A. and A.A.T. conceptualize of the study. A.A.M and A.O.A. were responsible for data curation and interpretation of data. Drafting of the article and revising it critically for important intellectual content was carried out by A.O.A., A.A.M, S.A. and A.A.T while final approval of the version to be submitted was done by all the authors.

Conflict of interest

The authors have no conflict of interest to declare.

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