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Research Article

Evaluation of Immunotherapeutic Effects of *Aloe vera* Polysaccharides Against Coccidiosis in Chicken

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Abstract

This study reports the immunotherapeutic effects of *Aloe (A.) vera* polysaccharides against coccidiosis in broiler chicken. For the purpose, polysaccharides were recovered from *A. vera* and analyzed by using HPLC. Three different hexose sugars including maltose, glucose and mannose were detected in hydrolyzed solution of *A. vera*. The extracted polysaccharides (graded doses) were evaluated for immunotherapeutic activities against coccidiosis in chicken. Results revealed that percent protection and daily weight gains were significantly higher (P<0.05) in chicken administered with *A. vera* polysaccharides as compared to control group. On the other hand, oocyst counts and lesion scores were lower (P<0.05) in polysaccharides administered chickens as compared to control. Moreover, anti-coccidial indices were also higher in chickens administered with polysaccharides (159.75-239.63) as compared to control (36.57). Except spleen, the organ-body weight ratios of all lymphoid organs of experimental and control groups were statistically similar (P>0.05). Based upon findings of this study, it was concluded that *A. vera* derived polysaccharides had immunotherapeutic activity against coccidiosis in chickens and might be further explored for its commercial feasibility for effective use in poultry industry to control avian coccidiosis.

Keywords: *Immunotherapeutic, Aloe vera , Polysaccharides, Coccidiosis, Chicken*

Tavuklarda Koksidiyoza Karşı *Aloe vera* Polisakkaritlerinin İmmunoterapötik Etkinliğinin Saptanması

Özet

Bu çalışma ile tavuklarda koksidiyoza karşı *Aloe (A.) vera* polisakkaritlerinin immunoterapötik etkisi rapor edilmektedir. Bu amaçla, *A. vera*'dan polisakkaritler elde edildi ve HPLC ile analizleri yapıldı. Maltoz, glikoz ve mannoz içeren üç farklı heksoz şekeri *A. vera*'nın hidrolize edilmiş solüsyonunda belirlendi. Ekstrakte edilen polisakkaritlerin (dereceli dozlarda) tavuklarda koksidiyoza karşı immunoterapötik etkisi araştırıldı. Sonuçlar; yüzde koruma ve günlük ağırlık kazanımının *A. vera* polisakkaritleri verilen tavuklarda kontrol grubuna oranla istatistiksel olarak daha yüksek olduğunu gösterdi (P<0.05). Oosit sayısı ve lezyon skoru ise polisakkarit verilen tavuklarda kontrol grubuna oranla istatistiksel olarak daha düşüktü (P<0.05). Anti koksidial belirtiler de polisakkarit verilen tavuklarda (159.75-239.63) kontrol grubuna oranla (36.57) daha yüksekti. Dalak dışındaki tüm lenfoid organların organ-vücut ağırlığı oranları deney ve kontrol grubundaki hayvanlarda benzerlik göstermekteydi (P>0.05). Çalışmanın bulgularına dayanılarak, *A. vera* polisakkaritlerinin tavuklarda koksidiyoza karşı immunoterapötik eksinin olduğu ve bu nedenle tavuklarda koksidiyozu kontrol altında tutabilmek amacıyla ticari kullanımının araştırılması gerektiği sonucuna varılmıştır.

Anahtar sözcükler: *İmmunoterapötik, Aloe vera, Polisakkaritler, Koksidiyozis, Tavuk*

INTRODUCTION

Coccidiosis is one of the most important protozoal infections of poultry industry, inflicting heavy economic losses in the form of high morbidity and mortality in affected flocks [1]. It is caused by different species of genus *Eimeria*, belonging to family *Eimeriidae*. Poor management such as damp litter, contaminated drinkers and feeders, high stock density and poor ventilation are the most important predisposing factors of this disease in intensive poultry production [2]. It has a negative impact on the production performance of affected birds in terms

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of retarded growth and poor feed conversion ratios in addition to high morbidity and mortality [3]. According to an estimate, it causes economic losses up to three billion US dollars annually worldwide [4,5].

In current era, the poultry industry largely relies upon the use of chemoprophylactic drugs and live vaccines to control coccidiosis [6,7]. At the same time, there is also increasing concern towards the use of alternative control measures due to some associated pitfalls of exiting control strategies including the emergence of drug resistant strains, high cost of vaccines and drug residues in meat and eggs [5,8]. At present, many alternative strategies are under investigation for effective, economical and environment friendly control of coccidiosis, including the use of medicinal plants [9,10]. In this context, *A. vera* has been reported for promising immunomodulatory effects in different animal models, highlighting it as a potential candidate for immunotherapy in different ailments [11]. *A. vera* is one of the most commonly used medicinal plants throughout the world with pronounced historical importance [12]. Its gel contains more than 75 active components including polysaccharides, minerals, phenolic compounds, proteins, sugars, vitamins, amino acids and saponins each with some pharmacological effects in different ailments [13,14]. Literature revealed that most of medicinal effects are due to polysaccharides present in inner leaf gel [15]. These polysaccharides had been extensively reported as a wound healing agent in different wound conditions by proliferation of fibroblasts and hydroxyproline and hyaluronic acid production in fibroblasts and thus extracellular remodeling in wound healing process [16]. It could inhibit inflammatory process by reduction of leukocytes adhesions and pro-inflammatory cytokines [17]. Its administration increases phagocytic and proliferative activity of reticuloendothelial system [18]. The *A. vera* polysaccharides are well documented for pharmacological activities in different animal disease models but only a few studies are available in the chicken model. Keeping in view, in continuation to our previous studies [12,19], this study aimed to investigate the immunotherapeutic efficacy of *A. vera* polysaccharides against coccidial infection in chickens.

MATERIAL and METHODS

Procurement and Processing of A. vera Leaves

Fresh leaves of *A. vera* were obtained from Botanical Garden, University of Agriculture, Faisalabad (UAF), Pakistan and their authenticity was confirmed from the concerned botanist of Department of Botany, UAF, Pakistan. The plant specimens were kept in the Ethnoveterinary Research and Development Centre, Department of Parasitology, UAF, Pakistan (Specimen Voucher No. 072). Fresh leaves after harvesting were subjected to surface sterilization by washing with chlorinated H_2O followed by formalin (0.005 ppm solution) and finally with distilled H_2O [19].

Separation of Leaf Gel

The mucilaginous leaf gel was separated from *A. vera* leaves within 3-4 h post collection to avoid aerodeterioration of gel contents. Briefly, the prewashed *A. vera* leaves were incised longitudinally with the help of a sharp sterilized knife followed by gentle scrapping of gel using a spatula. The gel was homogenized, filtered through cheese cloth and stored in screw capped jars at 4°C till further use.

Extraction and Hydrolysis of A. vera Polysaccharides

Polysaccharides were extracted from *A. vera* gel by following the methodology described by Chang et al.[20] with minor modifications. In brief, the *A. vera* gel was mixed with 95% ethanol (1:4) by vigorous shaking and incubated for 12 h at 4°C. The supernatant was discarded, and precipitate was subjected to centrifugation (6500×g for 10 min). The precipitate was mixed with dd-H_2O and incubated for 12 h and again precipitated with 95% ethanol (1:4). The procedure was repeated several times until all the colored material was removed. The final precipitate was mixed with dd-H_2O and treated with Sevag reagent [butanol:chloroform (1:4 v/v)]. The protein contents were removed by repeated oscillation and centrifugation procedures [21]. The deproteinated solution was mixed with 95% ethanol (1:3) to precipitate the polysaccharides. The precipitated polysaccharides were separated and subjected to further purification by washing 2-3 times with ethanol (absolute) followed by acetone and ethyl ether, respectively. Polysaccharides thus obtained were dried at 40°C for 24-48 h.

HPLC Analysis of Polysaccharides

The extracted polysaccharides were hydrolyzed to get the monomer units (monosaccharides) as described previously [22] with minor modifications. In brief, the polysaccharides were refluxed in trifloroacetic acid (2M; Sigma-Aldrich®, USA) at 100°C for 2 h in a round-bottom flask equipped with a reflux condenser. The TFA and water contents were removed by evaporation (75°C) and freeze drying (-65°C), respectively. The hydrolysed monosaccharides were analysed by using Shimadzu-10A HPLC workstation (Japan) equipped with a quaternary gradient pump unit and a refractive index detector (RID). The Rezex RCM-Monosaccharide Ca^{+2} column (Phenomenex, USA) was used to get absorption spectra at a wavelength of 235 nm at 80°C. Isocratic DD H_2O was used as mobile phase. Injection volume for each of monosaccharide standards and sample was taken as 20 µL.

The Infective Material

Sporulated oocysts of mixed *Eimeria (E.)* species including *E. tenella*, *E. acervulina*, *E. maxima* and *E. necatrix* maintained at Immunoparasitology Laboratory, UAF, Pakistan were used for this study to induce *Eimeria* infection in the birds. The infective dose was adjusted at the rate of 7×10^4

sporulated oocysts of per 2 mL of phosphate buffered saline (PBS).

Experimental Design

A total of 160 (one-day-old) broiler chicks were obtained from local market and reared under standard management conditions at Experimental Poultry Shed, Department of Parasitology, UAF. All the chicks were offered withdrawal feed and water *ad libitum* throughout the study and vaccinated according to the routine schedule against ND, IBD and HPS [5]. During the experimental trial, all procedures were performed in accordance to the guidelines of the Institutional Animal Care and Use Committee of UAF.

After 5 days of acclimatization, chicks were randomly divided into four equal groups A_1-A_4, each containing 40 chicks and were administered orally with graded doses of Aloe polysaccharides for three consecutive days i.e. 5th-7th days of age. Groups A_1, A_2 and A_3 were administered *A. vera* polysaccharides at the dose rates of 100, 200 and 300 mg.kg^{-1} body weight, respectively while group A_4 was kept on PBS as a control.

Immunotherapeutic Evaluation

On day 14th post-administration of *A. vera* polysaccharides, chickens of all the groups were challenged with infective dose of mixed species of genus *Eimeria* (local isolates; 7×10^4 sporulated oocysts per bird) with the help of an oral gavage [23]. In each group, chickens were monitored for oocysts per gram of faeces (OPG), daily weight gains, lesion scoring and mortality from day 3rd to 12th post challenge. For lesion scoring, dead and survived chickens in all the groups were killed humanely and scored for intensity of lesions as described by Johnson and Reid [24].

Further, the percent protection against lesions was calculated by using the formula described by Singh and Gill [25] as follows:

Per cent protection against lesions = (Average lesion score (IUG)-Average lesion score (IMG))/(Average lesion score (IUG)) × 100

Where,

IUG = Infected Untreated Group; IMG = Infected Medicated Group

Anti-coccidial Index (ACI)

Anti-coccidial index (ACI) was calculated to demonstrate the therapeutic efficacy of *A. vera* polysaccharides by following the formula described by Shah et al.[26] as follows:

Relative rate of weight gain was calculated by subtracting the body weight at the time of challenge from the body weight at the end of experiment. Survival rate was estimated by the number of survived chickens divided by the initial number of chickens. Lesion scores of the chickens from all groups were calculated by the method of Johnson and Reid [24] and oocyst value was calculated by using the formula described previously [27] as follows:

Development of Lymphoid Organs

The organ-body weight ratio of lymphoid organs including spleen, thymus, caecal tonsils and bursa of Fabricius were calculated on day 12th post challenge with *Eimeria* species. Briefly, chickens of all the groups were weighed individually. Thereafter, birds were killed humanely and their lymphoid organs were incised out and weighed. The results were expressed as percent organ-body weight ratios as described earlier [5].

Statistical Analysis

Data thus collected were analyzed by using statistical analysis software (SAS® 2004) through one-way ANOVA and Duncan's Multiple Range (DMR) test. The differences were considered significant at $P<0.05$.

RESULTS

HPLC Profile of A. vera Polysaccharides

HPLC analysis of the hydrolysed solution of *A. vera* polysaccharides revealed the presence of three different monosaccharide units including mannose, glucose and maltose at peak retention times (min) of 12.55, 11.08 and 9.423, respectively. Molar concentrations (%) of detected monosaccharides are presented in *Table 1*.

Immunotherapeutic Evaluation of A. vera Polysaccharides

- Oocyst Counts and Daily Weight Gains Post-Challenge: All the groups administered with graded doses of *A. vera* polysaccharides showed significantly lower ($P<0.05$) oocyst counts as compared to control from days 4th to 12th post challenge. Maximum OPG was recorded on day 9th post infection in all groups. OPG count was lower in group A_2 and A_3 as compared to A_1 and control. However, the

Table1. *Quantitative analysis of monosaccharides detected in the hydrolyzed solution of A. vera polysaccharides*

Monosaccharides	Retention Time (min)	Area (mV.s)	Height (mV)	Quantity (molar %)
Maltose	9.423	11.837	0.54	0.04
Glucose	11.08	35.252	0.605	0.11
Mannose	12.55	36.885	0.612	0.02

difference between groups A_2 and A_3 was statistically non-significant (P>0.05; *Fig. 1)*. On the other hand, daily weight gains were significantly higher (P<0.05) in chickens administered with different doses of *A. vera* polysaccharides as compared to those of control group; although no graded dose response was detected *(Fig. 2)*.

- Percent Protection, Lesion Scoring and Percent Protection Against Lesions: The highest protection (70%) was observed in group A_2 administered with *A. vera* polysaccharides (200 mg.kg^{-1} BW) followed by group A_3 (60%) and group A_1 (55%) and control group (30%). Chickens of all the groups (both survived and dead chickens) were examined for lesion scoring on a scale from 0 to 4. Chickens of experimental groups administered with *A. vera* polysaccharides showed lesser lesions and thus higher percent protection against lesions as compared to those of control group. Among experimental groups, chickens of group A_2 showed the lowest score of severe lesions followed by A_1 and A_3 as compared to chickens in control group, which showed severe lesion scores *(Table 2)*.

- Estimation of Anti-coccidial Index: The group A_2 administered with *A. vera* polysaccharides at the rate of 200 mg.kg^{-1} showed the highest anti-coccidial index (239.63) followed by those of groups A_3 (195.31) and A_1 (159.75). A value of 36.57 was also recorded for control group that could be due to the self-limiting nature of the *Eimeria* infection in poultry *(Table 3)*.

Organ-body Weight Ratios in *A. vera* Polysaccharides Administered and Control Groups: All with mixed *Emeria* groups showed statistically similar organ-body weight ratios (P>0.05) except spleen, which showed significantly higher (P<0.05) spleen-body ratio in birds of control group as compared to those administered with *A. vera* polysaccharides *(Table 4)*.

Table 2. *Lesion scoring and percent protection against lesions*

Group	Lesion Scoring of Birds					Protection Against Lesions (%)
	0	1	2	3	4	
Caeca						
A_1	0	5	6	11	18	23.75ab
A_2	0	8	14	6	12	36.25a
A_3	0	0	8	16	16	20b
A_4	0	0	5	11	28	3.25c
Intestine						
A_1	0	11	6	8	15	33.125b
A_2	0	12	9	5	9	49.375a
A_3	0	16	7	5	12	41.875a
A_4	0	0	2	16	22	12.5c

*Values sharing similar letters in a column are statistically non-significant (P>0.05); **A_1** = A. vera polysaccharides at dose rate of 100 mg.kg^{-1} BW; **A_2** = A. vera polysaccharides at dose rate of 200 mg.kg^{-1} BW; **A_3** = A. vera polysaccharides at dose rate of 300 mg.kg^{-1} BW; **A_4** = Control group*

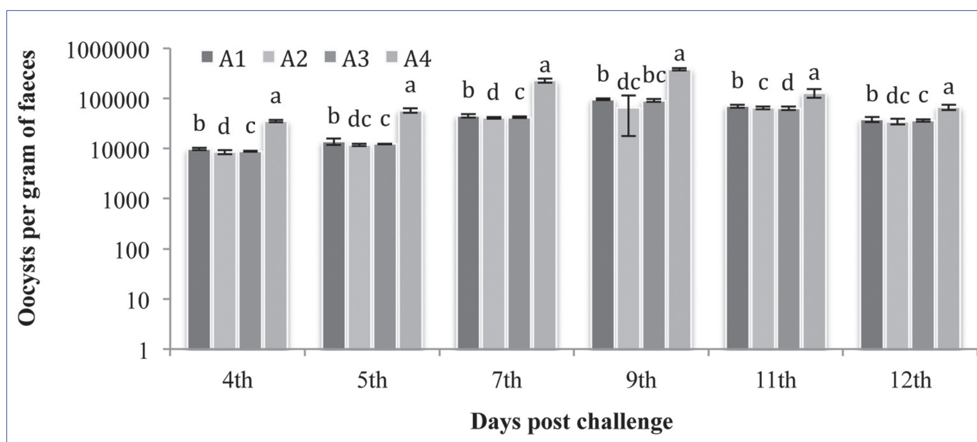

Fig 1. Oocysts per gram of faeces from day 4th to 12th post-challenge with *Eimeria* species. Bars sharing different letters on a particular day present a significant difference (P<0.05). A_1 = *A. vera* polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = *A. vera* polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = *A. vera* polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

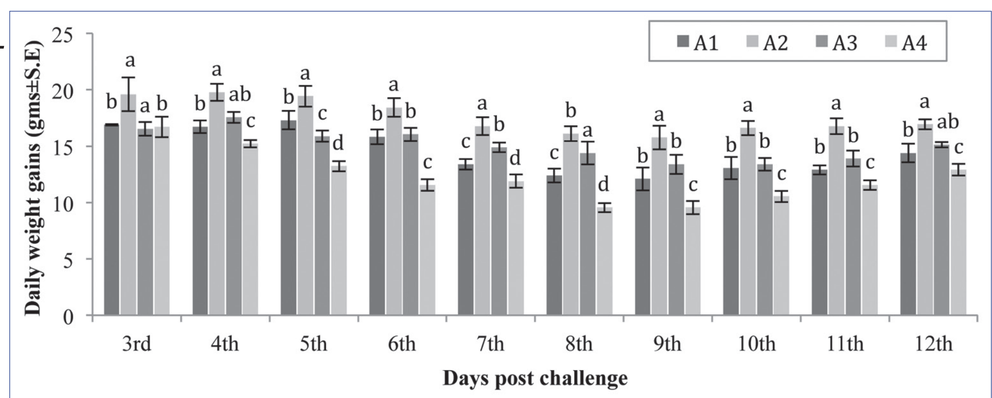

Fig 2. Daily weight gains of experimental and control chickens from day 3rd to 12th post challenge. Bars sharing different letters on a particular day present a significant difference (P<0.05). A_1 = *A. vera* polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = *A. vera* polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = *A. vera* polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

Table 3. *Anti-coccidial indices in experimental and control groups*

Groups	Relative Rate of Weight Gains	Survival Rate	Lesion Value	Oocyst Value	Anticoccidial Index
A_1	162.567	0.55	2.675	0.696	159.75
A_2	241.696	0.7	2.025	0.746	239.63
A_3	197.756	0.6	2.325	0.717	195.31
A_4	40.14	0.3	3.5	0	36.57

A_1 = A. vera polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = A. vera polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = A. vera polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

Table 4. *Organ-body weight ratio post challenge in experimental and control chickens*

Group	Thymus (Mean ± SE)	Spleen (Mean ± SE)	Bursa (Mean ± SE)	Caecal Tonsils (Mean ± SE)
A_1	0.37±0.01	0.27±0.02[c]	0.26±0.01	0.09±0.01
A_2	0.38±0.01	0.28±0.01[b]	0.25±0.01	0.08±0.01
A_3	0.37±0.01	0.28±0.01[b]	0.26±0.01	0.08±0.01
A_4	0.36±0.01	0.29±0.01[a]	0.25±0.01	0.07±0.01

Means sharing similar letters in a column are statistically non-significant (P>0.05); A_1 = A. vera polysaccharides given at rate of 100 mg.kg^{-1} BW; A_2 = A. vera polysaccharides given at rate of 200 mg.kg^{-1} BW; A_3 = A. vera polysaccharides given at rate of 300 mg.kg^{-1} BW; A_4 = Control group

DISCUSSION

Coccidiosis is an important protozoal infection of poultry of high economic importance having a negative impact on the production performance and thus farm profitability [4,28,29]. Conventionally, disease is controlled through medication and vaccination strategies but each with certain limitations. As an alternative approach, modern trends are molding towards the use of native biomolecules from different medicinal plants for the treatment of various ailments in both animals and human beings [10]. In this regard, *A. vera* reported to have significant immunoregulatory and immunostimulatory activities, mainly antioxidant effects; stimulation of phagocytes and humoral immunity in different animal models [12,30,31]. Keeping in view, this study was conducted to evaluate the immunotherapeutic efficacy of *A. vera* polysaccharides against coccidiosis in chicken.

Polysaccharides isolated from *A. vera* were analyzed by using HPLC. Results indicated the presence of three different monosaccharides including maltose, glucose and mannose. Previously, several polysaccharides including acemannan, arabinoxylan, arabinorhamnogalactan, galactan, galactogalacturan, galactoglucoarabinomannan, gluco-galactomannan, glucomannan and glucuronic acid had been isolated from different parts of *A. vera* plant [15,32]. Further, presence of saccharides including aldopentose, glactose, glucose, L-rhamnose and mannose in *A. vera* polysaccharides were also reported [33,34]. Tan et al.[13] analyzed *A. vera* polysaccharides by HPLC and reported presence of mannose as a monomeric unit of isolated polysaccharides. In other studies, different polysaccharides of variable molecular sizes have been isolated from *A. vera* [35,36] and this variability might be associated with isolation methodology along with seasonal and cultivational variations [34].

For immunotherapeutic evaluation of *A. vera* poly-saccharides, chickens of all the groups (experimental and control) were challenged with mixed *Eimeria* species in this study. Significantly lower oocyst counts in *A. vera* treated groups might be correlated to the development of resistance induced by *A. vera* polysaccharides against *Eimeria* species [12,37]. Yim et al.[38] also reported that *A. vera* extract can be used as a safe dietary supplement against coccidiosis. Some other studies had also reported the similar findings in broilers and rabbits [39-41]. Lesion score is the most common method for assessing intestinal condition during coccidiosis [42]. Chickens administered with polysaccharides, showed higher daily weight gains and lesser lesions on the caeca and intestine as compared to control. These lesser intestinal lesions might be due to the effects of *A. vera* on intestinal tract microflora, reduced bowel putrefaction that subsided/decreased inflammation [36] or lining of intestine layer with *Aloe* biomolecules [43]. Improved intestinal health in *A. vera* polysaccharides administered chickens might be responsible for better higher weight gains and thus better production performance [44].

Maximum protection (70%) in polysaccharides administered chickens might be correlated immunostimulatory activity of Aloe polysaccharides like acemannan which had been reported to reduce the opportunistic infections and stimulate wound healing [45]. Further, previous studies reported that carbohydrate polymers (glucomannans) present in *A. vera* played role in healing process [46] and inhibited cyclooxygenase pathway resulting in decreased prostaglandin production from arachidonic acids [47]. Vahedi et al.[48] reported that *A. vera* polysaccharides led to stimulate cellular and humoral immune responses by increased synthesis and release of T-lymphocytes and cytokines, which might be speculated to neutralize the pathogenic organisms like *Eimeria* species. Earlier, *A. vera* extracts administered at different dose rates had also revealed significantly elevated macrophages and white blood cell counts in mice [49]. Further, Cheesbrough [50] also reported *A. vera* polysaccharides to boost the activity of intestinal macrophages and T-lymphocytes up to 50 percent to prevent the penetration of pathogenic viruses,

bacteria and tumor cells. Results of present study also showed a similar response against coccidiosis in terms of higher survival percentage and reduced oocyst counts. Some previous studies on herbal biomolecules also reported the similar findings [5,10,28,45].

Anticoccidial index (ACI) reflects a comprehensive ability of any compound against coccidial infection. ACI values lower than 120 depict that compound/drug has no anti-coccidial activity; whereas values between 120 and 160 are considered partially effective but very effective at value > 160 [51]. In present study, *A. vera* polysaccharides administered at dose rates 200 and 300 mg.kg^{-1} of body weight showed ACI values higher than 160, so can be considered as very effective immunotherapeutic regimes against coccidiosis. *A. vera* polysaccharides did not show any significant effect on the development of different immune organs including thymus, caecal tonsils and bursa of Fabricius as compared to control. Only spleen-body weight ratios of chickens of control group showed significant difference from those administered with *A. vera* polysaccharides. Contrary to this, Darabighane et al.[45] reported a significantly higher relative weight of spleen in *A. vera* gel administered chickens; whereas, some previous similar studies reported a non-significant impact of herbal biomolecules on the development of lymphoid organs [5,12]. In this study, higher spleen-body weight ratio might be speculated due to cellular infiltration and spleen hypertrophy due to severity of coccidial infection in control group as compared to Aloe polysaccharide administered groups [52,53]. In conclusion, *A. vera* polysaccharids demonstrated the immunotherapeutic efficacy against coccidiosis in chickens and can be used successfully as a trustworthy alternative to anti-coccidial drugs, against which resistance has been emerged, to combat the avian coccidiosis.

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