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RESEARCH ARTICLE

Elucidating antiviral activity of *Curcuma longa* against H_9N_2 influenza virus using embryonated chicken egg model

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ABSTRACT

Curcumin is a potent antimicrobial herb used traditionally as a spice in culinary. This study was designed to evaluate the antiviral effects of curcuma longa extract against H_9 influenza virus. A total of 60 embryonated eggs were equally divided into 5 groups with 12 eggs in each group. Group 1 (G1) served as uninfected negative control. Whereas Group 2 (G2) was kept as positive control infected with known virus @ 0.2 ml with $10^{-9.3}$ $EID_{50.}$ Group 3 (G3) was offered *Curcuma longa* @ 0.2 mg/0.2 ml and H_9N_2 virus (@ 0.2 ml with $10^{-9.3}$ EID_{50}. Similarly, Group 4 (G4) was inoculated with extract of *Curcuma longa* @ 0.2 mg/0.2 ml per egg, whereas Group 5 (G5) was given Ribazole @ 0.2 ml/ egg. The crude extract and virus were administered on the 15th day of incubation and were checked after every 24 hours up to 96th hour post inoculation by random selection of 3 eggs. Death and survival rate were noted in all groups. Gross and histopathological lesions were also observed. Results revealed that *Curcuma longa* extract had significantly ($p<0.05$) reduced the mortality rate of embryos infected with H_9N_2 virus. In G3, increased lymphocytes and mild fatty changes were seen in liver. Whereas, mature RBCs, plasma cells and some lymphoblast's were observed in Spleen. Similarly, the bursa follicles were with lymphocytic aggregation. The G4 (*Curcuma longa*) and G5 (Ribazole) were with maximum embryo survival after 48 and 72 h post inoculation. This study revealed potential antiviral activity of *Curcuma longa* against H_9N_2 influenza viruses and can be opted as alternative to antibiotics and antiviral drugs to minimize the antimicrobial resistance in human and animal population.

Keywords: Poultry diseases; natural antiviral; drug resistance; ribazole.

INTRODUCTION

Avian influenza (AI) is a viral disease affecting respiratory, reproductive and digestive systems of domestic poultry (Bakeer *et al*., 2019). It belongs to *Orthomaxoviradae* family with negative strand and segmented RNA viruses and 15 different hemagglutinin (HA) and 9 neuraminidase (NA) recognized antigens (Tong *et al*., 2012).

Low pathogenic avian influenza infection can occur in all ages of birds with varying degree of mortality and morbidity. These viruses infect wild birds naturally and then disperse in domestic poultry birds. Primary infection may have 10-20% mortality but in secondary infection it reaches to 30-80% in flocks (Naeem *et al*., 2007). Subtype (H_9N_2) is non-highly pathogenic pathotype virus. Some frequent incidences with high mortality due to H_9N_2 have been observed in Iran and other Asian countries in broiler chickens. Lesions due to viral infection greatly vary due to type and age of the bird. Usually engorged blood vessels, Hemorrhages in trachea, proventricular and gizzard throughout the intestine can be seen. The gizzard mucosal lining may be removed easily. Breast muscle along with heart, abdominal and gizzard fat likely to show petechial hemorrhages (Tong *et al*., 2012).

Curcuma longa belong to family Zingiberaceae and is a perennial herb commonly cultivated in Indo Pak sub-continent. It contains several different secondary metabolites such as, curcuminoids, steroids and sesquiterpenes (Omosa *et al*., 2017). Curcumin is the main active ingredient and is well known for its use in food industry as preservative and colouring agent. It is also belived as potential antimicrobial, antioxidant and antiinflammatory agent (Han *et al*., 2018; Akbar *et al*., 2019; Praditya *et al*., 2019). It has also been tested previously against Chikungunya virus, Zika virus, human immunodeficiency virus (HIV) and influenza type A (Dai *et al*., 2018). Curcumin targets and interfere in viral attachment/ penetration (Chen *et al*., 2013). Currently, treatment of influenza virus infection is controlled through mass scale

vaccination of the flocks througout the world (Mohammed *et al*., 2020). Although amantadine is regularly being used in flocks against influenza A virus incubations, but increased resistance of common drugs draw the attension back on the uses of natural products agaisnt these visuses for control in poultry and other birds. This study have been designed to evaluate the antiviral potential of extract of *Curcuma longa* against influenza virus inoculation.

MATERIALS AND METHODS

Experimental design and area

This study was carried out in the Department of Pathology, University of Veterinary and Animal Sciences, Lahore. About 14 days old total of 60 embryonated eggs were divided into 5 equal (12 eggs) groups. Group 1 (G1) served as uninfected negative control. Whereas Group 2 (G2) was kept as positive control infected with known virus @ 0.2 ml with $10^{-9.3}$ EID_{50}. Group 3 (G3) was offered *Curcuma longa* @0.2 mg/0.2 ml and H_9N_2 virus (@ 0.2 ml with $10^{-9.3}$ EID_{50}. Similarly, Group 4 (G4) was inoculated with extract of *Curcuma longa* @ 0.2 mg/0.2 ml per egg, whereas Group 5 (G5) was given Ribazole @ 0.2 ml/ egg. (Table 1).

Extraction of plant

About 50 g ground powder of *Curcuma longa* was taken from phyto herbal shop were added into 70% ethanol and crude extract was collected using Soxhlet apparatus as described by (Fagbemi *et al*., 2009).

Virus Preparation

Virus was cultivated in chicken embryonated eggs followed by collection of allantoic fluid and lastly, titer of virus was checked by spot Hemagglutination test.

Eggs Inoculation

All the eggs (60) were inoculated with *curcuma longa* extract, virus and ribazole through the allantoic route. The G1 served as uninoculated control negative and G2 as positive control @ 0.2 ml virus with $10^{-9.3}$ EID_{50}.

The G3 was offered *Curcuma longa* @0.2 mg/0.2 ml and H_9N_2 virus (@ 0.2 ml with $10^{-9.3}$ EID_{50}. Similarly, Group 4 (G4) was inoculated with extract of *Curcuma longa* @ 0.2 mg/0.2 ml per egg, whereas Group 5 (G5) was given Ribazole @ 0.2 ml/ egg. Eggs were sealed and incubated at 37°C for few days and were regularly candled. The embryonic death was regularly observed up to 96 hours post inoculation. All the dead embryos were collected, chilled for 3h and then gross pathological changes were observed as proposed by (Sulaiman *et al*., 2011).

Histopathology

Different lymphatic tissues (spleen, bursa, Liver) were collected from embryos. A thin tissue section was stained with hematoxylin-eosin dye using histological paraffin embedding technique as suggested by (Khan *et al*., 2018).

Statistical Analysis

One way ANOVA was applied to the survival rate of embryos and p value calculation using SPSS software version. 20. The gross and histopathological lesions were measured by scoring method (Courtney *et al*., 2013).

RESULTS

In this study, 60 embryonated chicken eggs were randomly divided into 5 groups with 12 in each. Where 3 eggs from each group were selected at 48, 72 and 96 hours post inoculation for detailed observations. The embryonic survival rate was 100 % in G1 (negative control uninoculated group) and G4 (inoculated with extract of *Curcuma longa* @ 0.2 mg/0.2 ml per egg). Whereas G3 (Curcumin + virus) showed 75%, G5 (Ribazole + Virus) 83% and G2 (+ve control) 33.3% as least survival rate (Table 2; Figure 1). The chilled embryos were opened and washed with normal saline and checked for postmortem and gross pathological lesion (Table 3).

Histopathology

Liver

G1 exhibited mild congestion with fatty liver at 48th, 72nd and 96th hour post inoculation (HPI), in contrast to group 2, where severe congestion, was seen at 48 and 72 hours of inoculation.

Table 1. Experimental design

S#	Groups	No of Birds	Treatments/Inoculations
1	G1 (-ve control)	12	Uninfected, Negative Control
2	G2 (+ve control)	12	Infected with Known Virus, 0.2 ml virus with $10^{-9.3}$ EID_{50}
3	G3 (Curcumin + virus)	12	0.2 mg/0.2 ml each of Curcumin and virus with $10^{-9.3}$ EID_{50}
4	G4 (*C. longa*)	12	0.2 mg/0.2 ml
5	G5 (Rib+ Virus)	12	Ribazole @ 20µg/ml + 0.2 ml each of Curcumin and virus with $10^{-9.3}$ EID_{50}

Note: -ve = Negative, +ve = Positive.

Table 2. Embryonic death post inoculation

Hours Post Inoculation	G1 (-ve control)		G2 (+ve control)		G3 (Curcumin + virus)		G4 (Curcumin)		G5 (Rib+ Virus)	
	Live	Dead	Live	Dead	Live	Dead	Live	Dead	Live	Dead
24	3	0	2	1	3	0	3	0	3	0
48	3	0	1	2	3	0	3	0	3	0
72	3	0	1	2	2	1	3	0	2	1
96	3	0	0	3	1	2	3	0	2	1
Sum of live birds	12	0	4	8	9	3	12	0	10	2
Survival percentage (%)	100	33.3	75	100	83.3					

Note: -ve = Negative, +ve = Positive.

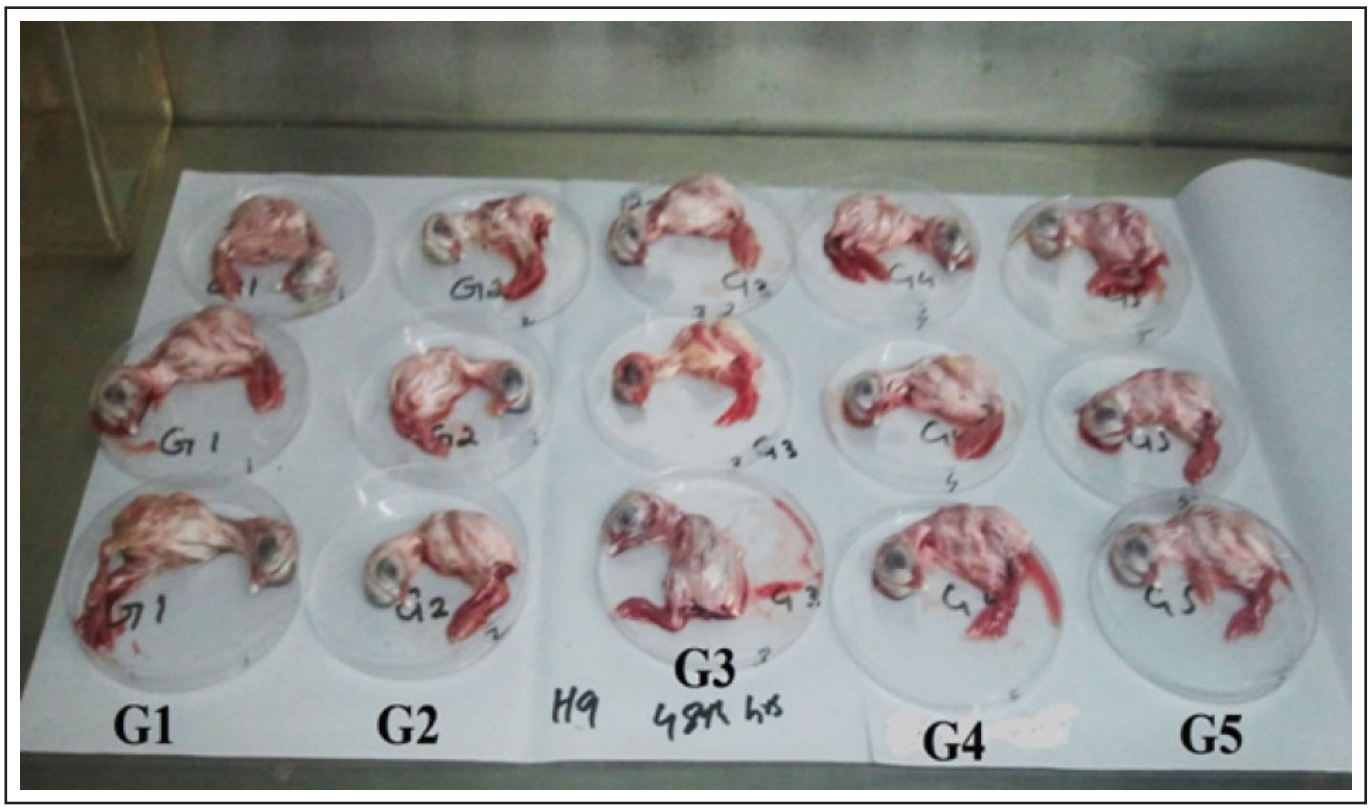

Figure 1. Embryos collected from all five groups 48 hours post inoculation.

Table 3. Gross pathological lesions in different organs of embryos, experimentally inoculated with virus

Group	HPI	Severity of Pathology in Selected Organs						
		Skeletal muscle	Thymus	Bursa	Spleen	Kidney	Liver	Lungs
G1 (-ve control)	48	–	–	–	–	–	–	–
	72	–	–	–	–	–	–	–
	96	–	–	–	–	–	–	–
G2 (+ve control)	48	+	+	–	+	–	+	+
	72	++	+	+++	++	+	++	++
	96	++	++	+++	+++	++	++	++
G3 (Curcumin+ virus)	48	+	–	–	+	–	+	+
	72	++	+	++++	++	++	++	+
	96	–	–	–	+	–	+	++
G4 (*C. longa*)	48	–	–	–	–	–	–	–
	72	+	–	–	+	–	–	–
	96	–	–	++++	+	+	–	–
G5 (Rib+ Virus)	48	+	–	–	+	–	+	+
	72	++	+	+++	++	++	+	+
	96	++	+	–	++	+	++	++

Legend: HPI = Hours Post Inoculation, – = no lesion, + = <20% congestion/hemorrhages, ++ = >50% sever congestion/hemorrhages, +++ = atrophy, ++++ = hypertrophy, Rib = Ribazole.

In G3 increased lymphocytes and mild fatty change was observed at 48 and 72 hours. Perivascular cuffing of plasma cells and hepatocytes degeneration was perceived after 96 hours post inoculation.

Similarly, in G4, Lymphocytosis, fatty changes and mild congestion was observed at 48th, 72nd and 96th hour of inoculation, while degeneration and congestion of hepatocytes was observed at 48th and 96th HPI in G5 (Figure 2).

Spleen

In G1 group, splenic arteries were congested. While, in G2 congestion, lymphoblasts were present predominantly and few lymphocytes were observed in 48th and 72nd HPI. In G3 mature RBCs with some lymphoblast and plasma cells was observed at 48th and 72nd HPI, at 96th HPI large no of RBC and lymphoblast cells were present. Necrosis and degeneration of few cells with karyolysed nuclei were seen. In G4,

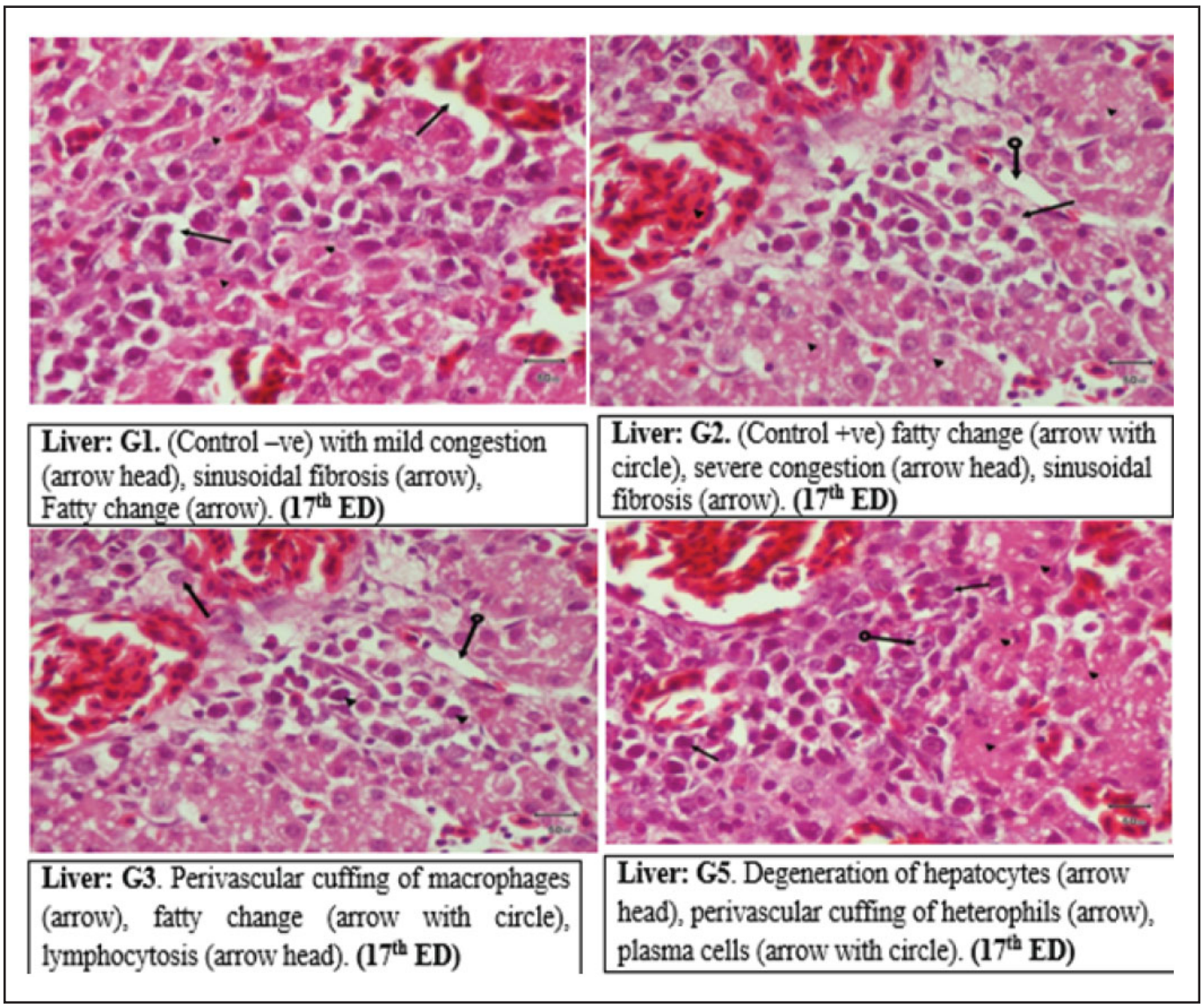

Figure 2. Histopathological changes observed in Liver of chicken of different groups under study with Hematoxylin and Eosin stain.

lymphoblast, pre lymphocyte and RBCs were observed in 72nd HPI. Similarly, in G5 in splenic parenchyma congestion degeneration and few necrotized cells were present. While at 96th HPI in few plasma cells RBC and lymphoblast were seen (Figure 3).

Bursa

In G1, normal bursa was seen while, in G2, under-developed with pseudostratified columnar epithelium bursal follicles were seen at 48th and 72nd HPI. In G3 developing bursa follicles with few lymphoblast and aggregation of lymphocytes were seen at 48th HPI. While at 96th HPI bursa was normally and fully developed. In G4 congested capillaries with large no of developed and developing follicles were observed after 72nd HPI. Similarly, in G5, bursa was found normal at 48th and 72nd HPI (Figure 4).

DISCUSSION

Curcumin is a very common medicinal herb used globally as culinary in addition to their ethno medicinal potential. It is used as dried powder and extract form. Generally, the extract is more potential as it contains dissolved bioactive compounds. It is used for various purposes against different infections both in animals and human beings. Influenza viruses cause severe morbidity and mortality and is a serious threat to the poultry industry. This study was designed in order to evaluate the potential of *curcumin* extract and ribazole (common antiviral drug) against H_9N_2 influenza virus. In this study embryonic survival rate was 100% in group 4 (Curcumin offered group) against H_9N_2 virus, while ribazole offered group G5 showed 83.3% survival. This explores the promising results of Curcumin extract as comparable with common antiviral drug. Our findings corroborate with some other researchers who also found curcumin with strong potential of anti-influenza activity (Chen *et al*., 2010; Xu *et al*., 2017; Dai *et al*., 2018; Richart *et al*., 2018; Shenge *et al*., 2021).

Moreover, Curcumin causes triggering of macrophage activation by inhibiting a specific NF-κB signaling pathway against influenza virus (Xu *et al*., 2017). This might be due the presence of some potent bio active components such as, curcuminoids etc. Various histopathological findings were observed in different organs *viz*, liver, Spleen and bursa after inoculation of curcuma longa and low pathogenic avian influenza virus in embryonated chicken model. Similarly (Namazi *et al*., 2010), have also performed their experimental

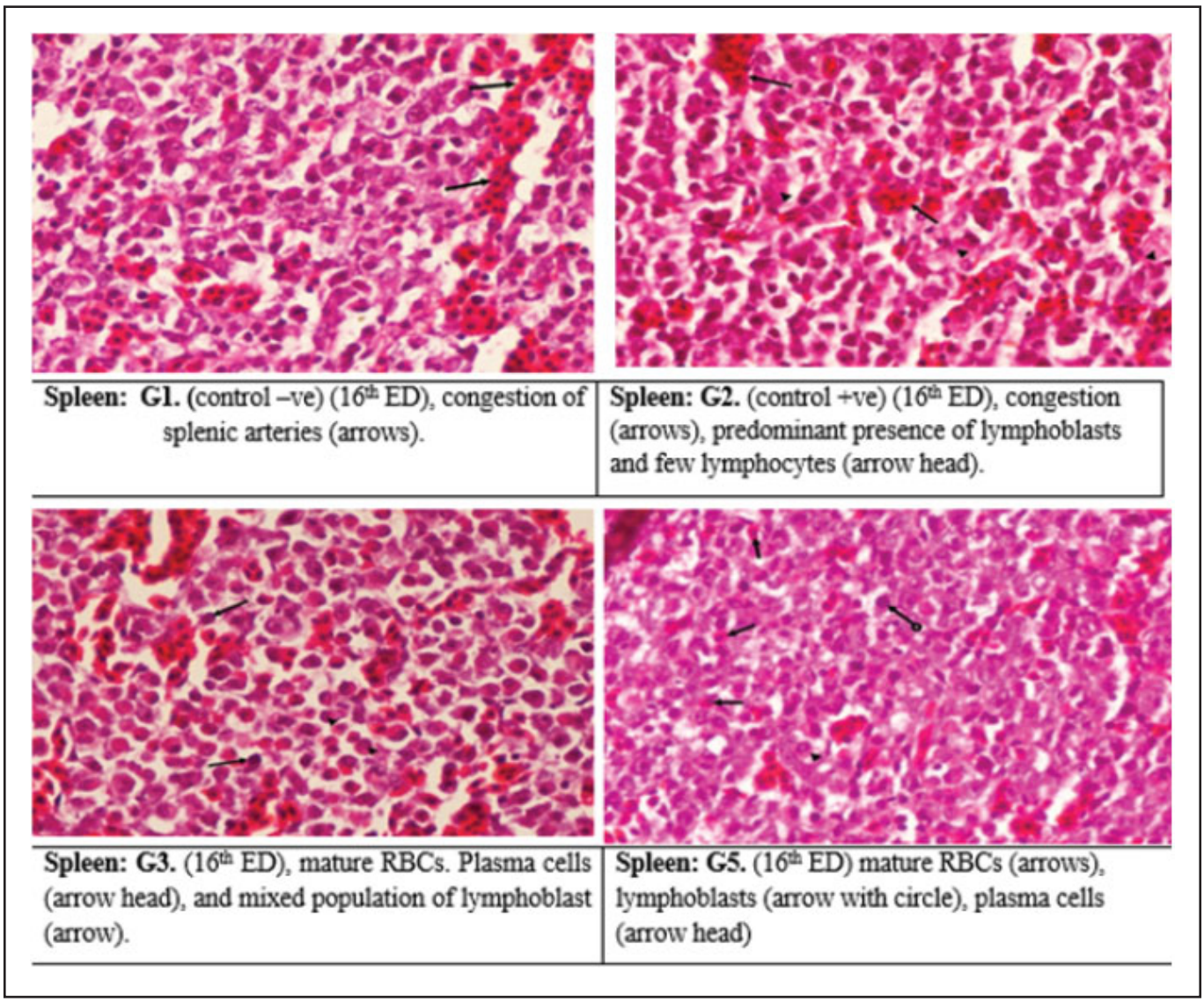

Figure 3. Histopathological changes observed in Spleen of chicken of different groups under study with Hematoxylin and Eosin stain.

module in embryonated egg and histopathology of brain and liver were performed. However, some researchers reported no mortality of embryos after inoculation of virulent viruses (Hablolvarid *et al*., 2013). In contrast embryos mortality was reported with H9N2 in broiler chicks in some other studies (Nili & Asasi, 2003). These differences in embryonic mortality may be due to differences in dose of the virus inoculated in different treatment groups.

In this study, supplementation of *Curcuma longa* extract was investigated in broiler chicken. In positive control group G2 necrosis, mild sinusoidal congestion, plasma cells, degeneration and mature RBCs were observed. These finding are in line with (Ono & Tuan, 1986), who also reported development of specific necrosis in hepatic tissues of chick and quail embryos.

Ribazole showed a significant decrease in viral load, decreased embryo mortality and histopathological changes in lymphatic organs. Similarly, significant decreased HA titer against avian influenza was reported (Wang *et al*., 2008). Ribazole is an approved antiviral drug generally used for the treatment of different viral hepatitis (Omer *et al*., 2014).

In virus inoculated group (G2, Positive Control) severe lymphocytic necrosis was noted in liver, spleen and bursa but these histopathological changes were markedly decreased in the *Curcuma longa* treated antiviral groups (G4). These findings are in line with (van Campen *et al*., 1989), who also reported effects of avian influenza virus on the lymphocytic structure and population in lymphoid organs (Bursa and Spleen) in term of histopathological changes, severe necrosis of splenic lymphocytes, as well as thymus and bursa. Similarly, (Ebrahimi *et al*., 2010) studied experimentally infected quails with H_9N_2 virus and found that liver, spleen, intestines and respiratory organs were affected severely.

CONCLUSION

In conclusion, ethanolic extract of *Curcuma longa* possesses strong anti-viral effect against avian influenza virus type H_9N_2. Mixing of *Curcuma longa* extract or powder with the feed or water will undoubtedly boost the immunity against influenza viruses and will surely help to use alternatives to anti infectious agents and boost the profitability of poultry farming.

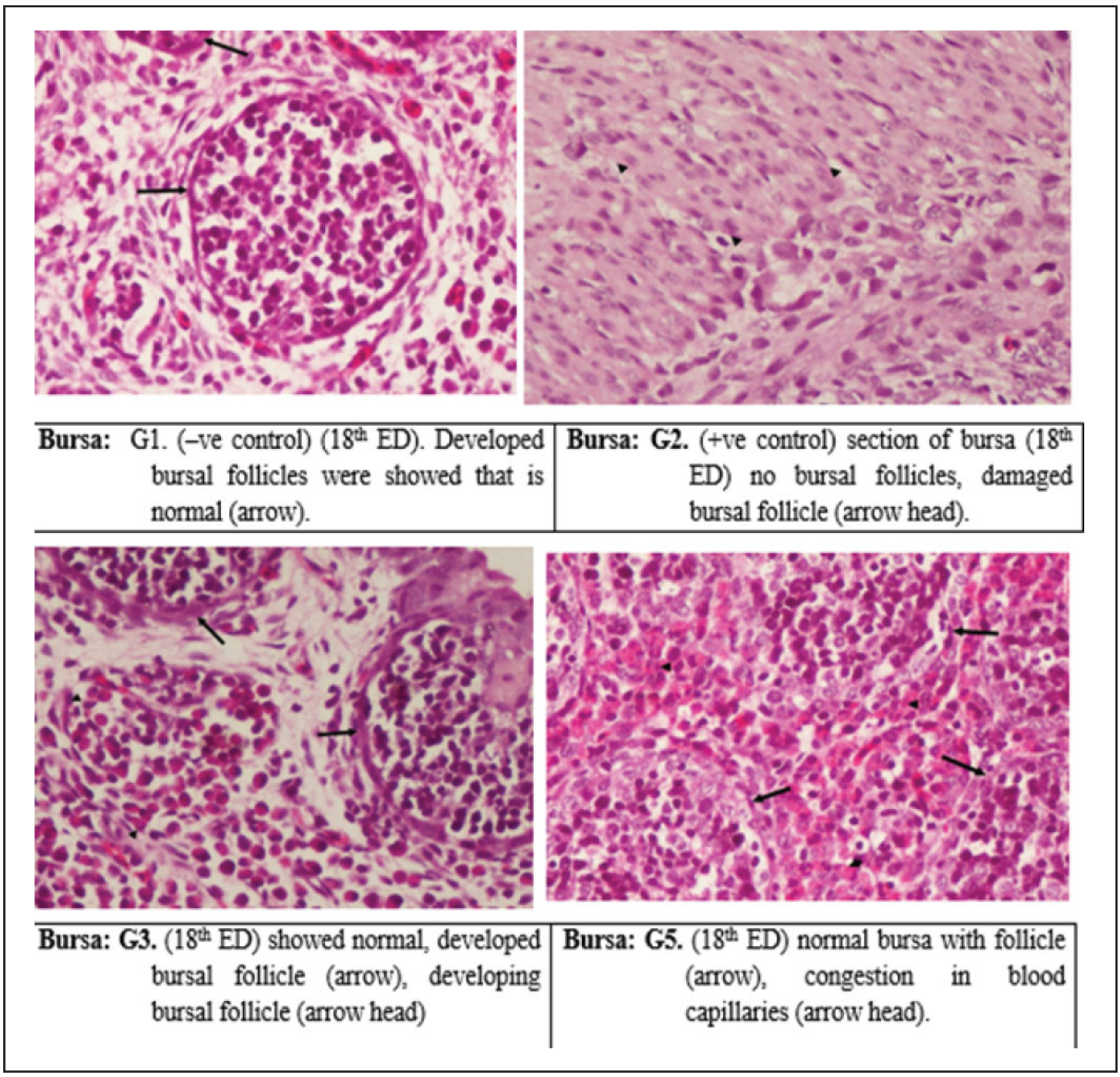

Figure 4. Histopathological changes observed in Bursa of chicken of different groups under study with Hematoxylin and Eosin stain.

Conflict of Interest

The authors declare that they have are no conflict of interests.

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