

## Evaluation of Antiviral Effect of Epigallocatechin Gallate, Epigallocatechin, Epicatechin Gallate and Green Tea Extract Against Fowl Adenovirus-4

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**Abstract.-** Green tea possesses various biological and pharmacological activities. Green tea because of its ingredients catechins, possesses strong antiviral activity. The present study was designed to evaluate the antiviral efficiency of green tea extract (GTE), epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and epigallocatechin (EGC) *in-vitro* through cell culture and *in vivo* in broiler chickens against the challenge of fowl adenovirus type 4 (FAdV-4). Green tea extract was found most promising antiviral agent *in-vitro* having selective index (SI) 3.195 µg/ml with minimum cell toxicity towards normal cells. Green tea extract showed maximum protection in broiler chicks against challenge *in vivo* with a survival rate of more than 90% at a dose rate of 100 mg/ml. Gross and histopathological lesion score was minimum in GTE treated group followed by EGCG, EGC and ECG.

**Key words:** Green tea, catechins, fowl adenovirus Type-4, broilers, antiviral effects.

### INTRODUCTION

Adenoviruses are concerned with a range of human and animal pathologies (Doronin *et al.*, 2001). The fowl adenoviruses (FAdVs) are mostly accountable for naturally acquired epidemics of inclusion body hepatitis/hydropericardium syndrome (IBH-HPS), gizzard erosions and respiratory tract disease (Marek *et al.*, 2010). These are heterogeneous viruses categorized into five different types (A-E) and twelve serotypes (FAdV-1 to 12) (Benko *et al.*, 2005). Non-enveloped, icosahedral FAdV-4 of the group 1 adenovirus genus in the family Adenoviridae is defined as the disease causing agent for IBH-HPS (Kumar and Chandra, 2004; Aslam *et al.*, 2012). The fowl adenoviruses are non-enveloped, 70-90 nm in diameter single linear, double stranded DNA virus, which have a characteristic icosahedral capsid of 240 non vertex capsomers (hexon) and 12 vertex capsomers (penton) each with one or two fibers protruding from the penton base (Taharaguchi *et al.*, 2012).

The pathogenic lesions of IBH-HPS consist of clear or straw-colored fluid in the pericardial sac with inflammation of liver and kidney, and mortality ranging from 30 to 70% (Hafez, 2011; Anjum *et al.*, 1989). Kim *et al.* (2009) observed basophilic intranuclear inclusion bodies in hepatocytes and circular, clear necrotic foci in liver. FAdVs can be transmitted vertically through the embryonated eggs and horizontally through personnel, fomites and transport, which play important roles in the spread of virus (Alemnesh *et al.*, 2012). FAdV-4 interacts with the immune system of birds causing suppression of humoral and cellular responses (Singh *et al.*, 2006).

Proteolytic dispensation is an indispensable part of many viruses life cycle. Adenoviruses bear a highly precise cleavage site on seven viral proteins and two cellular proteins with an encoded cysteine protease (Weber, 1995).

One of the most imperative economic objectives in the poultry industry is to minimize losses caused by infectious diseases (Fingerut *et al.*, 2003). Antiviral medications are not approved for use in animals due to drug resistance concern (Ilyushina *et al.*, 2005) and these drugs are very expensive to administer to livestock animals (Lee *et*

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*al.*, 2012). These concerns emphasize the urgent need to develop the antiviral compounds appropriate for animals and poultry.

Green tea (*Camellia sinensis*, Theaceae) is one of the most popular beverages in the globe and is produced from the buds of the *Camellia sinensis* plant (Schramm, 2013; Hsu *et al.*, 2011). The polyphenolic compounds known as catechins are the major active ingredients in green tea (Balentine *et al.*, 1997). These and other compounds present in green tea may activate the defense system against bacteria, fungi and viruses (Friedman, 2007). Due to the extensive use of green tea, the potential biological effects have been studied both *in vitro* and *in vivo* and hence green tea has gained significant attention as an agent that could reduce the risk of several diseases (Jin, 2013). The green tea, which is non-fermented that is steamed or pan fried and dried to inactivate the enzyme (Cabrera *et al.*, 2006). The natural catechin components in green tea are epigallocatechin-3-gallate, epicatechin, epigallocatechin, epicatechin-3-gallate and galloocatechin gallate (Wang *et al.*, 2003). Various biological and pharmacological properties of green tea and its major constituent polyphenols include anti-oxidative (Higdon and Frei, 2003), antibacterial (Araghizadeh, *et al.*, 2013; Steinmann *et al.*, 2013), anticancer (Bode and Dong, 2009) and antiviral activities (Oh *et al.*, 2013; He *et al.*, 2011). The galloyl group present in catechins inhibits the endonuclease activity of viral RNA polymerase of influenza virus (Kuzuhara *et al.*, 2009). Adenovirus maturation, infectivity and dismantling solely responsible on the suitable adenain activity, which suggested that target for the development of antiviral agent is the viral protease of adenovirus (Mangel *et al.*, 2001). Green tea itself and epigallocatechin-3-gallate have antiviral properties *in vitro* against human adenovirus type 2 at several levels through direct inactivation of the virus particles, inhibition of intracellular virus growth and viral protease, adenain (Weber *et al.*, 2003).

The present paper reports antiviral activity of green tea and its isolated catechins against the fowl adenovirus type-4 through *in vitro* in cell culture and *in vivo* in broiler chickens against IBH-HPS virus challenge.

## MATERIALS AND METHODS

### *Cell culture and virus infection*

The Vero cells were propagated as monolayers in complete Dulbecco's medium containing 10% fetal bovine serum and supplemented with penicillin and streptomycin (10 unit/ml, Biowest). The fowl adenovirus type 4 was obtained from the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. The virus was isolated from an outbreak of inclusion body hepatitis hydropericardium syndrome, which was confirmed through PCR, and nucleotide sequence and submitted to GenBank with Accession No. DQ 264728 (Mansoor *et al.*, 2009). The virus was grown and titrated in Vero cells by plaque assay, by means of Vero cells overlaid with 1% low melting agar in usual growth media. The cells were fixed with 1% (w/v) crystal violet after 5 days of infection. To determine the virus titer, the plaques were counted.

### *Chemicals and reagents*

Green tea extract (GTE) was prepared by addition of 20ml boiling double distilled deionized water ( $d_3H_2O$ ) to one g of dry tea leaves for ten min at 75°C in a sterilized and sealed glass container, was centrifuged and filtered through a 0.22 $\mu$  filter. Green tea extract was lyophilized for further use.

The green tea catechins, namely epigallocatechin gallate (EGCG; Cat #4143), epicatechin gallate (ECG) (Cat #3893) and epigallocatechin (EGC) (Cat #3768) were purchased from Sigma-Aldrich.

### *Plaque reduction assay*

Confluent monolayer of Vero cells cultured in 24 well tissue culture plate were infected with 100 plaque forming units (PFU) of FAdV-4 (Das *et al.*, 1999). After allowing adsorption of virus at 37°C for 2 h, the viral inoculum were decanted and the cells were washed with Hank's balanced salt solution. Overlay agar medium contained 0, 50, 100, 200  $\mu$ g/ml of GTE while ECGC, EGC and ECG were mixed @ 0, 30, 60 and 120  $\mu$ M/ml.

Incubation of the cultures was done for 3 days at 37°C in the presence of 5% CO<sub>2</sub> and the monolayer was fixed by using 4% formaldehyde

solution for 30 min. The agarose was removed by rinsing water and was stained by using 1% (w/v) crystal violet. The antiviral activity was determined by following formula:

$$\text{Percentage Inhibition} = 1 - \frac{\text{number of plaque tested}}{\text{number of plaque control}} \times 100$$

The obligatory minimal concentration of different compounds to restrain the formation of virus plaque number by 50% ( $EC_{50}$ ) were calculated by regression analysis of the dose-response curves produced from the data according to Cheng *et al.* (2002).

#### *Virus yield reduction assay*

Vero cells were grown as monolayers in 12 well tissue culture plates. At the confluence, the medium was decanted and monolayers was infected with  $10^6$  pfu of FAdV-4 per well. For the virus adsorption, the plates were placed on the shaker for 45 min at room temperature for regular overturning in drug-free conditions. The viral inoculum was removed and replaced with the medium containing various concentrations of test compounds (Song *et al.*, 2005). Viruses were harvested at 8, 24 and 36 h post-infection and the virus yield was estimated by plaque assay on Vero cells. As a control, the infected cells incubated in test compounds free medium were included during the whole experiment.

#### *Cytotoxicity assay*

Vero cells were grown at a concentration of  $5 \times 10^4$  cells in 96-well plate for 24 h. The medium was replaced with medium containing different concentrations of test compounds and cells were further incubated for 48h. Cell viability was determined by the MTT 3-(4,5-dimethylthiazol-2-yl) 3,5 diphenyl tetrazolium bromide (Bibasic) assay (Liu *et al.*, 2003) in which mitochondrial succinate dehydrogenase activity converts the tetrazolium salt (MTT) to the dark-blue formazan. The culture medium was aspirated and 100  $\mu$ L of MTT solution (2 mg/ml) was added to each well followed by incubation for 4h. The dark formazans were dissolved in DMSO and absorbance of each well was measured at 540 with ELISA reader

(Biotek, USA). The median cellular cytotoxicity concentration ( $CC_{50}$ ) was the concentration of the compounds that resulted in the death of 50% of the Vero cells.

Data were calculated as the percentage of inhibition by the following formula.

$$\text{Inhibition \%} = 100 - \frac{OD_t}{OD_s} \times 100$$

Where,  $OD_t$  and  $OD_s$  indicates the optical density of the test substances and the solvent control, respectively. The concentration of 50% cellular cytotoxicity ( $CC_{50}$ ) of test substances was calculated.

#### *Selectivity index evaluation*

The selectivity index (SI) of green tea extracts and its isolated compound against FAdV-4 was evaluated as the ratio of  $CC_{50}$  to  $EC_{50}$ .

#### *Antiviral activity in vivo*

Three hundred and twenty day-old specific pathogen free (SPF) broiler chicks were obtained from Sabir's poultry Breeders, Pakistan and reared under standard husbandry conditions in an experimental animal house of the Institute of Microbiology following the guideline of the Animal Ethics committee, University of Agriculture, Faisalabad, Pakistan. On 14<sup>th</sup> day of age, the birds were divided into four even groups (A, B, C and D); each group was divided into four sub group each (A1, A2, A3, A4, B1 .... and D4) having 20 birds. The EGCG, ECG and EGC were given orally 30, 60 and 120 mg/ml of drinking water to first three subgroups of each of A, B and C, respectively. GTE was administered at the dose of 50, 100 and 150 mg/ml orally to subgroups D1, D2 and D3, respectively. The subgroups A4, B4, C4 and D4 were kept as negative control. The treatments were given to all the groups after the challenge with fowl adenovirus type 4 having  $10^{4.8}$  mean egg infective dose ( $EID_{50}$ ). Starting from the day of challenge the treatments were continued for 6 days. During the treatment period, all the groups were examined daily for mortality and clinical signs. Survival rate and body weight of the birds were evaluated. Gross lesions of the necropsies birds were examined on liver, spleen and kidney for the presence of lesions.

The weights of liver, spleen and kidney were recorded and organ to a body weight ratio was calculated (Khan *et al.*, 2010). Tissue samples were fixed in 10% neutral buffered formalin. Section of 5  $\mu\text{m}$  thickness was processed for staining with hematoxylin and eosin for histopathological examination (Bancroft and Gabble, 2008) and scored from 0 to 3 based upon severity of the lesions.

### Statistical analysis

Student's unpaired *t*-test was used to assess the difference between the test sample and untreated control. A *P* of  $<0.05$  was considered statistically significant. One-way ANOVA was employed to evaluate the difference of organ to body weight ratios, gross lesion scores and histopathological lesion scoring among different test samples. SPSS soft was used for statistical analysis.

## RESULTS

### GTE cytotoxicity

The percentage inhibition of the Vero cells was dependent on the concentrations of green tea extract and its derivatives. It was measured by MTT assay as shown in results that EGCG, ECG and EGC was tolerated by the cells at concentration of 30, 60 and 120  $\mu\text{M}/\text{ml}$ , respectively whereas 180 and 240  $\mu\text{M}/\text{ml}$  EGCG, EGC and EGC were toxic to normal cells (Fig. 1A) with  $\text{CC}_{50}$  59.56, 70.88 and 34.95  $\mu\text{M}/\text{ml}$ . GTE was less toxic to cells at concentration 50-250  $\mu\text{g}/\text{ml}$  Fig. 1B, while  $\text{CC}_{50}$  of GTE was 100.23  $\mu\text{g}/\text{ml}$ . Therefore, the antiviral activity of EGCG, EGC and ECG was assayed at 120  $\mu\text{M}/\text{ml}$  or lower and GTE was assayed at concentration of 150  $\mu\text{g}/\text{ml}$  or lower.

Table I shows effective concentration ( $\text{EC}_{50}$ ), cellular cytotoxicity ( $\text{CC}_{50}$ ) and selective index (ratio of  $\text{CC}_{50}$  value/ $\text{EC}_{50}$  value).

### Anti-viral activity of GTE

MTT assay provided some initial clinical data that was used for antiviral activity against fowl adenovirus type-4 through plaque inhibition assay in Vero cells. As shown in (Fig. 2), EGCG, EGC and

ECG inhibited  $36.8421 \pm 2.10526$ ,  $28.7719 \pm 3.21584$  and  $27.7193 \pm 3.21584\%$  at 120  $\mu\text{M}/\text{ml}$  with mean 50% inhibitory concentration ( $\text{EC}_{50}$ ) 207.12, 241.70 and 392.52  $\mu\text{M}/\text{ml}$ , respectively as compared to control. GTE inhibited the viral replication at 100 and 150  $\mu\text{g}/\text{ml}$  at the rate of  $77.7583 \pm 2.90658$  and  $62.4561 \pm 4.25416\%$ , respectively with an  $\text{EC}_{50}$  of 34.80  $\mu\text{g}/\text{ml}$ . GTE inhibited the plaques more efficiently as compared to EGCG, EGC and ECG.

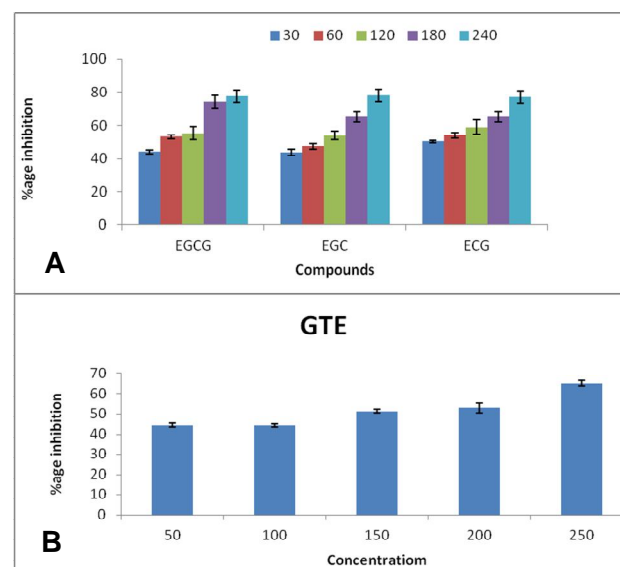


Fig. 1. A, overall cell inhibition % after treatment of normal cell with EGCG, EGC and EGC at (30 to 240  $\mu\text{M}/\text{ml}$ ). B, Over all cell inhibition % after treatment of normal cell with GTE at 50 to 250  $\mu\text{g}/\text{ml}$  was determined by MTT assay in Vero cells. Data represented as mean  $\pm$  standard deviation of triplicates.

Table I - Anti-adenovirus activities and cytotoxicities of the EGCG, EGC, EGC and GTE.

Compounds	$\text{EC}_{50}$ <sup>a</sup>	$\text{CC}_{50}$ <sup>b</sup>	SI <sup>c</sup>
EGCG	207.12	59.56	0.28
EGC	241.70	70.88	0.32
ECG	392.52	34.95	0.089
GTE	34.80	100.23	3.165

a,  $\text{EC}_{50}$  represents the concentration of test compound necessary for a reduction in plaque number by 50% relative to control without test compound; b,  $\text{CC}_{50}$  represents cellular toxicity to uninfected Vero cells as determined by MTT assay; c, SI is the ratio of  $\text{CC}_{50}$  values to  $\text{EC}_{50}$  values.

**Table II.- Relative organ weights (percent of the body weight) of broiler treated with different compounds at various concentrations of liver, spleen and bursa and liver gross lesions.**

Treatment	Control	Organ to body weight ration ( $\mu\text{M}/\text{mL}$ )			Overall mean	P value
		30	60	120		
<b>EGCG</b>						
Liver	6.10 $\pm$ 0.16d	7.8 $\pm$ 0.19a	7.38 $\pm$ 0.13b	6.90 $\pm$ 0.10c	7.04 $\pm$ 0.66	0.000
Bursa	0.35 $\pm$ 0.01a	0.22 $\pm$ 0.0c	0.28 $\pm$ 0.004b	0.29 $\pm$ 0.014b	0.28 $\pm$ 0.50	0.000
Spleen	0.18 $\pm$ 0.01a	0.10 $\pm$ 0.0c	0.13 $\pm$ 0.008b	0.12 $\pm$ 0.007b	0.13 $\pm$ 0.03	0.000
Lesion score	0.00 $\pm$ 0.00c	3.80 $\pm$ 0.45a	3.60 $\pm$ 0.55a	2.80 $\pm$ 0.45b	2.55 $\pm$ 1.61	0.000
<b>EGC</b>						
Liver	6.10 $\pm$ 0.16	6.14 $\pm$ 3.16	7.38 $\pm$ 0.19	6.98 $\pm$ 0.19	6.65 $\pm$ 1.56	0.516
Bursa	0.35 $\pm$ 0.01a	0.24 $\pm$ 0.02c	0.29 $\pm$ 0.01b	0.31 $\pm$ 0.01b	0.30 $\pm$ 0.04	0.000
Spleen	0.18 $\pm$ 0.01a	0.11 $\pm$ 0.007c	0.13 $\pm$ 0.008b	0.15 $\pm$ 0.01b	0.14 $\pm$ 0.02	0.000
Lesion score	0.00 $\pm$ 0.00b	3.60 $\pm$ 0.55a	3.40 $\pm$ 0.55a	3.40 $\pm$ 0.54a	2.60 $\pm$ 1.60	0.000
<b>ECG</b>						
Liver	6.10 $\pm$ 0.16c	6.76 $\pm$ 0.16b	6.86 $\pm$ 0.11ab	7.04 $\pm$ 0.16a	6.69 $\pm$ 0.39	0.000
Bursa	0.35 $\pm$ 0.01	0.33 $\pm$ 0.01	0.31 $\pm$ 0.02	0.32 $\pm$ 0.01	0.33 $\pm$ 0.02	0.057
Spleen	0.18 $\pm$ 0.01a	0.1480 $\pm$ 0.01ab	0.13 $\pm$ 0.02b	0.14 $\pm$ 0.01ab	0.15 $\pm$ 0.02	0.008
Lesion score	0.00 $\pm$ 0.00b	2.60 $\pm$ 0.55a	2.80 $\pm$ 0.84a	3.00 $\pm$ 0.71a	2.10 $\pm$ 1.37	0.000
<b>GTE</b>						
Liver	6.10 $\pm$ 0.15bc	6.44 $\pm$ 0.11a	6.06 $\pm$ 0.11c	6.30 $\pm$ 0.10ab	6.22 $\pm$ 0.19	0.000
Bursa	0.35 $\pm$ 0.01	0.34 $\pm$ 0.02	0.35 $\pm$ 0.02	0.32 $\pm$ 0.02	0.34 $\pm$ 0.20	0.076
Spleen	0.18 $\pm$ 0.01a	0.14 $\pm$ 0.01b	0.17 $\pm$ 0.01a	0.15 $\pm$ 0.01ab	0.16 $\pm$ 0.02	0.005
Lesion score	0.00 $\pm$ 0.00c	2.20 $\pm$ 0.45a	0.20 $\pm$ 0.45b	0.60 $\pm$ 0.55b	0.75 $\pm$ 0.97	0.000

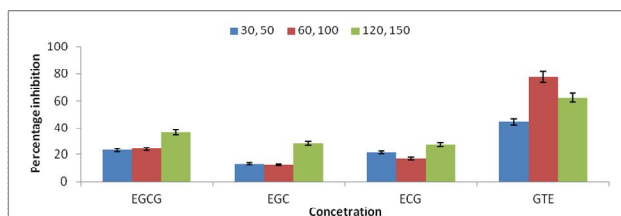


Fig. 2. Percentage inhibition of plaques by all tested compounds at 30, 60 and 120  $\mu\text{M}/\text{mL}$  of EGCG, EGC, ECG and 50  $\mu\text{g}/\text{mL}$ , 100  $\mu\text{g}/\text{mL}$  and 150  $\mu\text{g}/\text{mL}$  concentrations of GTE mix with 2% low melting agarose and medium on Vero cells after infected with 100 pfu/ml of FAdV-4. For each test compound percentage plaque formation ratio relative to control was determined.

Virus yield reduction assay was performed to analyze the effect of GTE and its derivative at different times after infection in Vero cells. The cells were infected with  $10^6$  pfu/ml of virus. At 8, 24 and 36 hours of post-infection virus yields were determined by plaque assay on Vero cells. As shown in Figure 3, the virus yields were reduced on

Vero cells by 2-10 log units depending on the concentration of the compounds. The maximum decrease in virus yields was observed at 120  $\mu\text{M}/\text{mL}$  of EGCG, EGC and ECG (we observed at 24 and 36 h of post-infection). GTE was shown to reduce the maximum virus yields at the concentrations of 100 and 150  $\mu\text{g}/\text{mL}$  given for 24 to 36h post-infection. The results showed that GTE was very efficient against the tested virus. The results further support the previous data on plaque assay.

#### *In vivo antiviral activity in broilers chickens*

##### *Survival rate*

Highest survival rate was observed in the birds treated with GTE followed by EGCG then EGC, which was followed by ECG as shown in Figure 4. Among different concentrations of green tea extract, 100 mg/ml gave maximum survival rate followed by 150 and 50 mg/ml.

##### *Organ to weight ratio and gross lesions in liver*

Relative organ weight (percent of the body

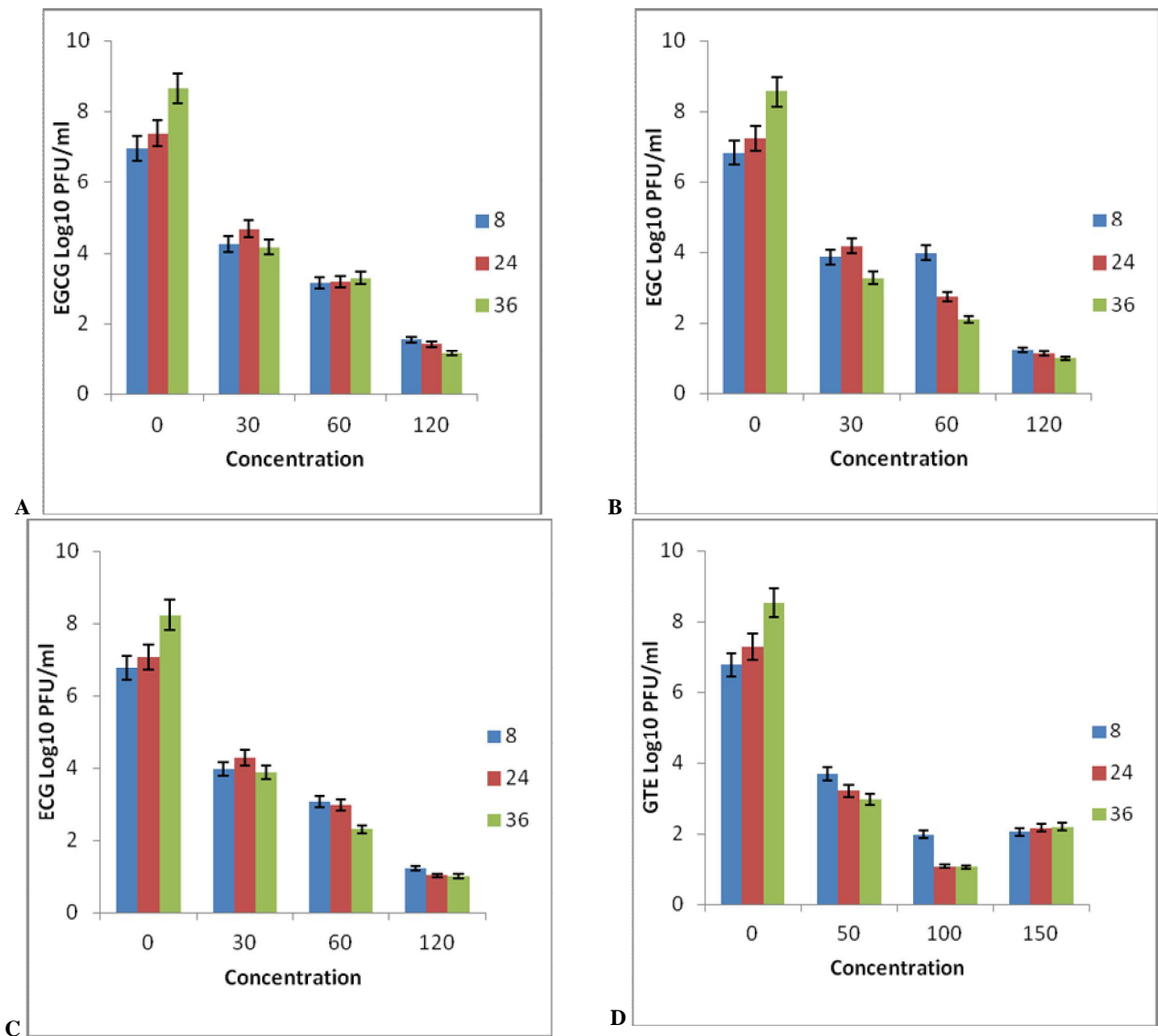


Fig.3. Inhibitory effect of catechins and GTE on Vero cells in 12 well plates infected with  $10^6$  pfu/ml of FAdV-4. Culture medium was harvested at 8, 24 and 36 (hrs) post-infection. The viral yield was estimated by plaque assay on Vero cells. A, EGCG; B; EGC; C; ECG and D; GTE

weight) of broiler chicks after the treatment of test compounds is shown in Table II. Relative organ weight of liver, bursa and spleen were higher than the control group. Among all the groups GTE treated groups shown higher relative organ weight as compared to EGCG, EGC and ECG. Liver gross lesions were observed in all the treated groups, which showed significant reduction in lesions in GTE treated groups compared to control and EGCG, EGC and ECG treated groups.

#### *Histopathological lesions scoring*

Histopathological lesions (Fig. 6) were observed in all groups treated at different concentration of GTE and green tea derivatives with a range of severities after challenge with FAdV-4 as shown in Figure 5. The severity of lesions was markedly reduced in GTE treated group at 100 mg/ml as compared to control observed in liver, kidney and bursa. Cumulative scores of liver, kidney and bursa of birds treated with EGCG, EGC

and ECG were significantly higher, while the group treated with GTE was significantly lower as compared to other groups.

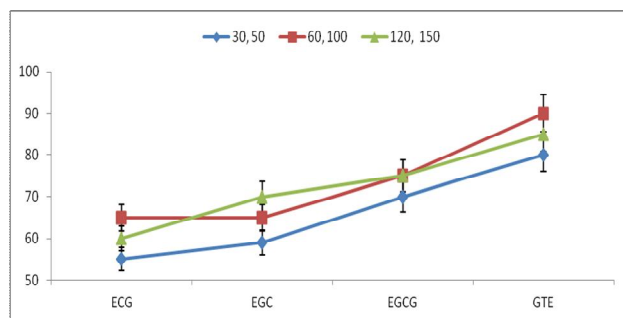


Fig.4. Survival rate of Broiler chickens after treatment with different compounds EGCG, ECG and EGC at (30 to 240 µM/ ml) and GTE at 50, 100 and 150 µg/ ml) and by giving challenge with FAdV-4

## DISCUSSION

Viral infections are the foremost human and animal diseases, which have a considerable economic impact (Cook and Kalt, 2010). Antiviral agents of plant origin are easily accessible, mostly nontoxic and inexpensive (Vahabpour-Roudsari *et al.*, 2007). Some of them pose the broad-spectrum antiviral activity as ideal candidates in antiviral therapy (Mukhtar *et al.*, 2008). During the last decade, several novel diseases, which affect the poultry with variable consequences, have emerged. Among these, IBH-HPS has a significant position (Kataria *et al.*, 2006). Green tea in the Asian population is a historically popular beverage and produced from the leaves of evergreen *Camellia sinensis* plant. The polyphenolic compounds known as catechins are the major active ingredients in green tea (Balentine *et al.*, 1997). These and other compounds present in green tea may interact with the defense system against bacteria, fungi and viruses (Friedman, 2007).

In the present study, *in vitro* assays were recognized and employed to perceive the antiviral effects of GTE and its isolated compounds against FAdV-4. The results of Cytotoxicity assay showed that on Vero cells of tested compounds were dose dependent; higher concentration of the compounds was significantly toxic to the cells that were

consistent with the previous results of Liu *et al.* (2003). The compounds were selected for antiviral assay, which was less toxic to the cell having 30, 60 and 120 µM of EGCG, EGC and ECG and in case of GTE 50, 100 and 150 µg/ml was selected. The green tea catechins, particularly EGCG reported to be more toxic to cancerous cells than the normal cells (Morre *et al.*, 2000).

Antiviral activity was observed through plaque inhibition assay on Vero cells. The most promising activity was observed in the GTE treated group, which inhibit the maximum plaque number at 100 µg/ml followed by 150 and 50 µg/ml as compared to control with EC<sub>50</sub> of 30.80 µg/ml. Our results were in line with Weber *et al.* (2003), who observed that GTE and EGCG inhibited the cellular process against the adenovirus. The Active ingredient of green tea EGCG not only inhibits the cellular processes but also promotes the release virus from the cell. Song *et al.* (2005) noted that the polyphenolic mixture was more efficacious than a single compound against the influenza virus followed by EGCG.

Virus yield reduction assay was performed on Vero cell to calculate the virus yield by plaque assay at various time intervals at 8, 24 and 36 hours. Results of virus yield reduction assay of ECG and EGC was shown same as EGCG the maximum reduction was observed at 120 µM of these two compounds that was followed by 60 and 30 µM with control. Virus yield assay of GTE against FAdV-4 maximum reduction in virus yield was at 100 µg 24 and 36 hour post infection followed by 150 and 50 µg. GTE shown maximum inhibition of virus on plaque assay after harvesting the virus at various time's intervals and reduced the virus yield at dose dependent manner. However, these all conferred antiviral effects on all stages of the infectious cycle. These results of virus yield reduction assay coincided with the finding of Song *et al.* (2005), who observed that isolated compounds EGCG was the most effective antiviral compounds against the influenza virus, and ECG was the less effective compound among the tested compounds. They observed that polyphenolic mixture was very effective at all stages of the virus infectious cycle. The complete inhibition influenza virus was observed at 120 µM concentration of EGCG.

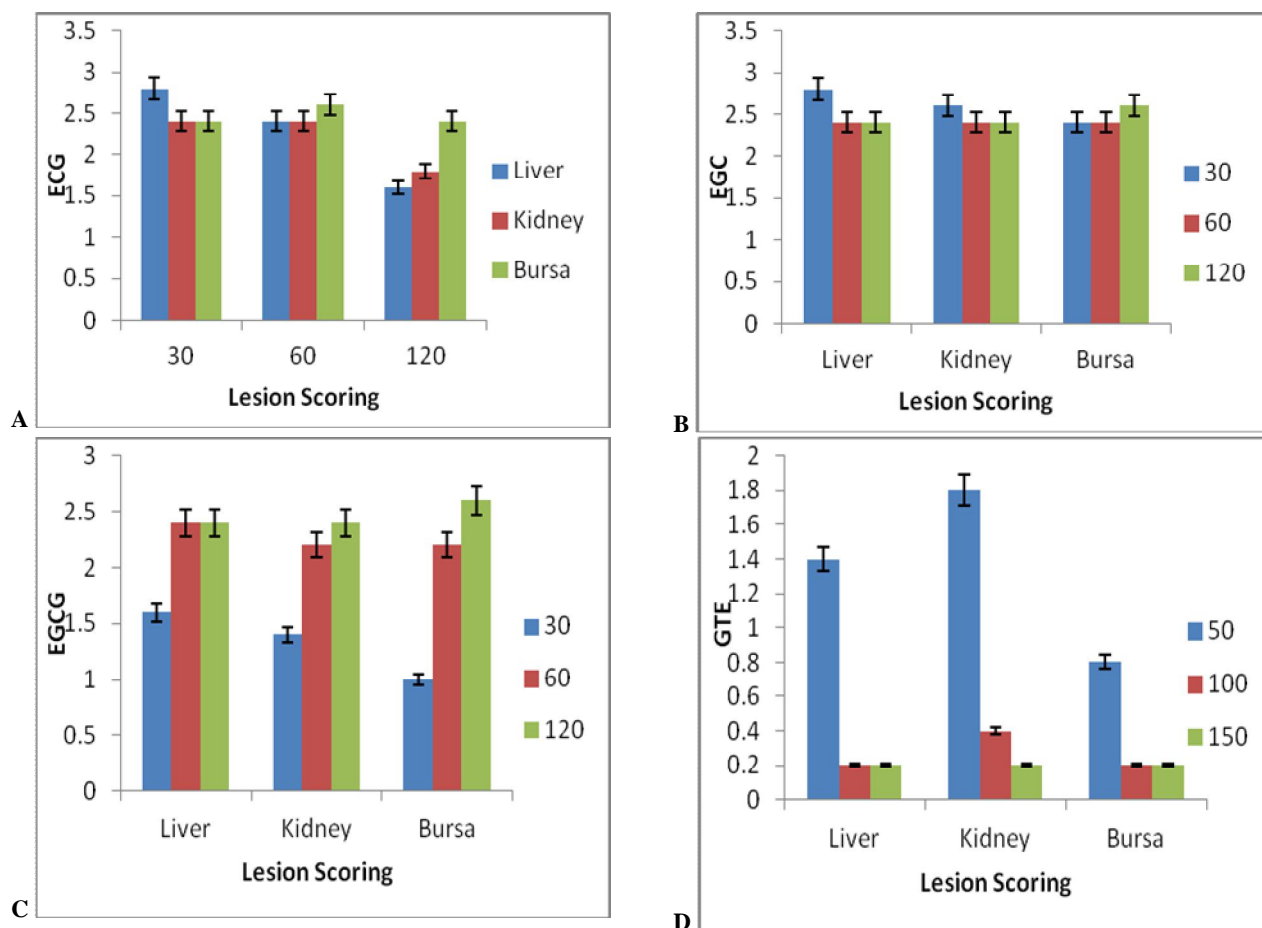


Fig.5. Histopathological Lesion scoring of Liver, Bursa and Spleen at various concentrations of different compounds; **A, B and C** groups were treated with EGCG, ECG and EGC at (30, 60 and 120 µM/ml) whereas group **D** were treated with GTE at 50, 100 and 150 µg/ml). The histopathological scoring is reflected in the figure 5 A, B, C and D.

*In vitro* study showed that the GTE was more efficient than other compounds, which were confirmed in another finding due to the presence of other active components of GTE (Xu *et al.*, 2008). The  $EC_{50}$  of GTE 34.80 and  $CC_{50}$  was 100.23 with a selective index (SI) 3.165 that results were shown that high (SI) showing maximum antiviral activity with minimum cellular toxicity according to the guide line for the evaluation of the antiviral drugs. These results agreed Oh *et al.* (2013) who evaluated antiviral activity of green tea against the feline calicivirus (FVS) having  $EC_{50}$  values 0.13 mg/ml and indicated that anti-FCV activity with lowest concentration and  $CC_{50}$  18.57 of GTE extract was on the top of the test compounds means that it has

strong antiviral activity with low toxicity.

The morbidity and survival rate of the GTE group were significantly higher compared to control group that is also in line with results of Noguchi *et al.* (2008). The gross lesions were higher in infected untreated control, which include the lesions in kidney, liver and the spleen of birds. The GTE treated group showed no gross and histopathological lesions having no symptom of hydropericardium and there was no enlargement of liver. The gross and histopathological lesions in GTE treated groups were followed by EGCG, EGC and ECG groups with mild lesions observed in each of the treated group. These findings were according to the finding of Barbour *et al.* (2007), who evaluated the GTE



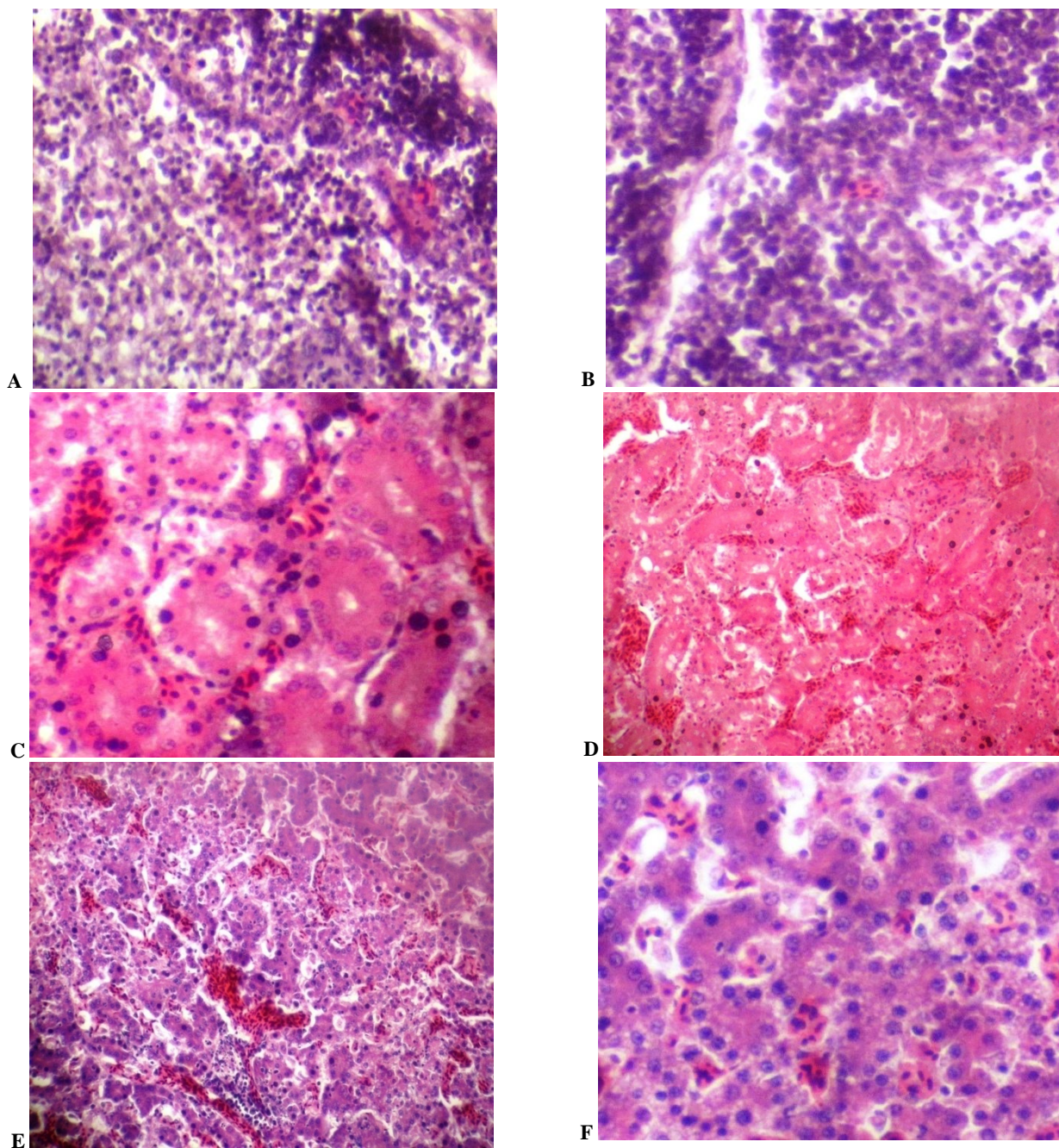


Fig. 6. Histological structure of Bursa of Fabricius (A, B), kidney (C, D), and liver (E, F) of fowl; **A**, severe lymphoid depletion of bursa of Fabricius of bird treated with 30 mg/ml of ECG; **B**, Mild lymphoid depletion of bursa of Fabricius of bird treated with 100 mg/ml of GTE; **C**, Severe congestion, hemorrhage and cell infiltration of kidney of the bird treated with 30 mg/ml of ECG; **D**, mild degenerative changes in kidney of birds treated with 100 mg/ml of GTE; **E**, liver mononuclear cell infiltration with severe congestion and hemorrhage of the bird treated with 30 mg/ml of ECG; **F**, liver of the birds treated with 100 mg/ml of GTE.

against influenza virus challenge in chickens. The tea polyphenolic compounds have antiviral activity

against viruses has been already reported in different studies. However, Barbour *et al.* (2007)

studied antiviral effects of GTE *in vivo* against avian influenza virus only (Lee *et al.*, 2012). The GTE treated group showed 90% protection of broiler chicks at 100 mg/ml followed by 150 and 50 mg/ml and in case of isolated compounds EGCG, EGC, and for ECG, 120 mg/ml, which was determined as effective dose. The body weight gain in different groups was also compared statistically. Maximum weight gain was observed in GTE treated group (100mg/ml) birds as compared to control groups that coincides with the finding of Sriram *et al.* (2008), 20 mg/ml of EGCG decrease lipid peroxidation, body weight was significantly improved, enzymatic and non enzymatic antioxidant status was enhanced due to its antioxidant properties. ECG treated group gave statistically significant difference of organ to the body weight ratio at all concentration. In EGC treated group, difference in liver to the body weight ratio was statistically non significant, but spleen and bursa weight ratio was statistically different at all concentration. In EGCG treated group spleen and liver weight ratio were significant statistically as compared to control but the bursa to the body weight ratio was not significant. In GTE treated group liver to the body weight ratio at 100 mg/ml was statistically non significant in comparison with control and bursa to the body weight ratio had statistically significant difference at all concentration, but spleen to the body weight ratio was statistically non significant at 100 mg/ml concentration of GTE. From above discussion, it was concluded that GTE at 100 mg/ml concentration gave maximum protection against challenge with FAdV-4. The GTE maintained the original appurtenance of tested tissues, which was in accordance with the finding of Gawish *et al.* (2010). It was observed the green tea extract against nicotine toxicity that restores the original appearance of testicular tissue. Regarding its affect on liver, GTE produced minimum gross, pathological and histopathological lesions, which was in agreement with the finding of Avwioro *et al.* (2010) who observed that GTE had no adverse effects on the histology and biomarker of liver. GTE showed maximum antiviral activity both *in-vitro* in cell culture and *in vivo* setting against challenge in broiler chickens. These data provide the scientific

basis of GTE used in chickens.

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