Evaluation of antiviral effect of Epigallocatechin gallate, Epigallocatechin, Epicatechin gallate and Green tea extract against fowl adenovirus-4

By

Azhar Aslam
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IN

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“Dedication”

THE WORK IS DEDICATED TO MY LATE MOTHER

WHO ALWAYS INSPIRED ME TO DREAM BIG
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Up and above everything else, I bow my head before Almighty “Allah” alone, the most gracious and compassionate, the omnipotent, the only creator of the whole universe; whose bounteous and exaltation flourished my thoughts and thrived my ambitions to eventually shape up the cherished fruit of my modest endeavors in the form of this manuscript. Next to Allah, all praises to His Prophet “Hazart Muhammad” (Peace Be upon Him), the most perfect and exalted among and ever borne on the surface of earth, whose eternal teachings would remain a source of guidance and inspiration for mankind forever.

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<td>FBS</td>
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<td>HTS</td>
<td>High throughput screening</td>
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<td>Inclusion body hepatitis hydropericardium syndrome</td>
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<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
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<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide</td>
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<td>NF-κB</td>
<td>Nuclear factor Kappa-light-enhancer activated B cells</td>
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<td>pTP</td>
<td>precursor terminal protein</td>
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<td>RCS</td>
<td>Reactive Carbonyl species</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RPHA</td>
<td>Reverse passive haemagglutination</td>
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<td>SBP</td>
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<td>SI</td>
<td>Selective index</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>SPF</td>
<td>Specific Pathogen Free</td>
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Abstract

Tea is the second most consumed drink globally after water. Green tea is the non-fermented form of tea prepared from the buds of *Camellia senensis* plant. Green tea extract (GTE) and its isolated compounds epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and epigallocatechin (EGC) were evaluated for their capacity to inhibit fowl adenovirus type 4 (FAvD-4) *in-vitro* through plaque reduction assay, its virucidal effect and its cytotoxicity was evaluated to normal cells through 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) assay. *In-vivo* study was conducted in broiler chicks to evaluate its protective efficacy against challenge of FAvD-4. Plaque reduction assay GTE at dose rate of 100mg/ml was shown maximum inhibition of plaque *in-vitro* followed by EGCG, EGC and ECG. In virus reduction assay, the test compounds were added to infected cells and harvest the virus at 8, 24 and 36 hours of post infection. The harvested virus was subjected to plaque assay and maximum inhibition in virus yield was observed at 100 mg/ml of GTE at 24 hours of post infection against FAvD-4 with (EC$_{50}$) 34.80µg with a 50% CC$_{50}$ 109.23µg having SI 3.195 showed strong antiviral effects with minimum CC$_{50}$ toward normal cells. Maximum cell inhibition was shown by EGCG, EGC, and ECG at 120 µM and 180 µM and 200 µM respectively while GTE showed minimum cell inhibition at different concentration.

*In-vivo* study, GTE was shown maximum protection in broiler chicks against challenge which was shown survival rate of more than 90% at 100 mg/ml GTE. Gross and histopathological lesion score was minimum in GTE treated group followed by EGCG, EGC and ECG. GTE was showed promising antiviral activity against FAvD-4 in cell culture which was evaluated in *in-vivo* and was a candidate agent to uaed in poultry for this virus infection.
INTRODUCTION

Viruses are intracellular pathogens and make use of their hosts’ intracellular machinery to replicate. Viral diseases are easier said than done to treat and only five drugs were present for the treatment of viral diseases as little as a decade ago (De Clercq, 2001). Presently, more than 30 antiviral drugs approved and numerous others drugs are in advanced phase III clinical trials due to better understanding of life cycle of different viruses and virus specific events for the targets of antiviral drugs (De Clercq, 2001). Approved antiviral drugs, more than 50% are for human immunodeficiency virus (HIV) treatment; the remaining is mostly for influenza virus, herpesvirus and hepatitis B virus (HBV). (De Clercq, 2002). Regrettably, present treatment of viral diseases are always not well tolerated and there is deficiency of effective treatment for a number of important viral infections. There is dire need for additional refinement of antiviral drug design and development to highlight these deficiencies. Furthermore, recently plant extracts having antiviral prospective against resistant viral strains to antiviral agent’s conventionally use to treat viral infections (Tolo et al., 2006) have confronted the modern drug discovery practices, and consider very careful look toward the study of medicinal plants having natural antiviral components.

Adenoviruses are incriminated in an array of human and animal pathologies (Whickam, 2000), infecting man, animals and birds. A little attempt has been intended for the development of antiviral strategies (Pang et al., 2001). According to the International Committee on Taxonomy of Viruses (ICTV) has divided the member of the family adenoviridiae in five genera: Mastadenovirus, Atadenovirus, Aviadenoviruss, Siadenovirus and Ichtadenovirus based on genomoic organization, virion properties, serological, structure and size difference (Benkő et al., 2005).

There are at least 10 structural proteins present in Adenoviruses. Adenoviral soluble antigens contained different genus-subgenus and type-
specific epitopes. The icosahedral particles (70-90 nm diameters) of soluble antigen collected of 252 capsomeres amid of single linear double-stranded DNA molecule as a genome. The capsomeres are composed of 240 hexons and 12 pentons having one or two fibers (Taharaguchi et al., 2012).

Aviadenovirus are discrete from other genera having two fibers per vertex (Laver et al., 1971; Gelderblom and Maichle-Lauppe, 1982), and only infect birds (Benkő et al., 2005). The genus Aviadenovirus in the family Adenoviridae is known as Fowl adenoviruses (FAdV’s). The FAdV’s are mostly accountable for naturally acquire epidemic of inclusion body hepatitis/hydropericardium syndrome (IBH-HPS), gizzard erosions and respiratory tract disease (Marek et al., 2010). FAdV’s are heterogeneous viruses and categorized in to five different types (A-E) and twelve serotypes (FAdV-1 to 12) (Benkő et al., 2005). In general, the widespread presence of antibody titre and isolation of FAdV’s from chickens which is clinically healthy that argues the major role of FAdV’s in a definite poultry disease (Monreal, 1984). In the late eighties, the presence of virulent FAdV’s changed this view that some viruses from the FAdV serotype 4 and 8 involve in disease production (Schonewille et al., 2008). Non–enveloped, icosahedral FAdV-4 of the group 1 adenovirus genus in the family Adenoviridae was defined as the disease causing agent for IBH-HPS (Mazaheri et al., 1988; Kumar and Chandra, 2004). In some cases, each condition is observed separately; however, recently two conditions are often seen as a whole, so the name of IBH-HPS is widely used to describe a pathological condition (Hafez, 2011). The pathogonomic lesions of IBH-HPS consist of clear or straw-colored fluid in the pericardial sac with inflammation of liver, kidney, and mortality ranging from 30% to 70% (Anjum et al., 1989). IBH-HPS has caused huge economic losses to the poultry industry in Pakistan since September 1987 when it was reported at Aggara Goth, the extensive broiler growing area in Karachi, Pakistan (Azhar et al., 2012). Kim et al., (2009) observed basophilic intranuclear inclusion bodies in hepatocytes and circular, clear necrotic foci in liver. FAdV’s can be spread vertically through the embryonated eggs and
horizontally through personnel fomites and transport which play important role in the spread of virus (Aleme
nesh et al., 2012). FAdV-4 interacts with the immune system of birds. The virulent avian adenoviruses have
immunosuppressant effect on humoral and cellular immune responses and also reported for other serotypes as well (Singh et al., 2006).

Proteolytic dispensation is an indispensable part of many viruses life cycle. Adenoviruses (AdVs) bear a highly precise cleavage site on seven viral proteins and two cellular proteins with an encoded cysteine protease (Weber, 1995). Infectivity of virus is enormously dependent on the proteolytic function (Greber et al., 1996). Suitable adenain activity is responsible for maturation, infection and dismantles of adenovirus, is dependent on proper adenain activity, suggesting that appropriate target for the development of antiviral agents is the viral protease of adenovirus (Weber, 2003; Mangel et al., 2001; Pang et al., 2001). The adenovirus protease is sensitive to a variety of protease inhibitors, such as GTP’s and papain (Weber et al., 2003).

Tea is the most well-liked consumed drink in the globe subsequent to water (Steinmann et al., 2013). The leaves of two cultivated plants, usually used to produced the tea which is known as Camellia sinensis (L) O. Kuntze var. sinensis and Camellia sinensis var. assamica (Masters) Kitamura (Theaceae) (Saha et al., 2010). Tea classified into three most important kinds depending on manufacturing procedure; green tea, which is non-fermented that is steamed or panfried and dried to inactivate its enzyme, fermented black and red tea and semi-fermented oolong tea (Cabrera et al., 2006). In addition, green tea possesses reported human health benefits by its pleasing flavor and aroma (Yang and Landau, 2000). The therapeutic belongings of tea leaves have been recognized for thousands of years (Yokoyama et al., 2004). Leaves of the plant Camelia sinensis produce the tea, which have two distinctive bioactive compounds, flavonoids and methylxanthines (Balentine et al., 1997). A group of alkaloids methylxanthines are in small quantities including caffeine, theobromine, and theophylline (Manning and Roberts, 2003). Most of the
valuable effects of green tea attributed to its polyphenolic compounds catechins that comprise 25 to 35% which is flavonols, a subtype of flavonoids (Meltzer et al., 2009; Ahn et al., 2003). Green tea is characterized by the existence of high amount of polyphenolic compounds known as catechins chemically (Liu et al., 2013). In polyphenols, catechins belong to the flavan-3-ol group of flavonoids, Green tea catechins are thermo stable and stable in acidic circumstance while those are unstable in alkali and neutral condition (Matsumoto et al., 2012). Early work on the beneficial effects of green tea is made in Japan and China because it is preferred in these countries due to local traditions. Of the tea produced worldwide, 78% is black tea, that is regularly consumed in the Western countries, 20% is green tea, which is frequently consumed in Asian countries, and 2% is Oolong tea which is made (by partial fermentation), mostly in southern China. Brewed tea includes several compounds, especially polyphenols, and various studies have shown that compounds present in tea diminish the risk of a variety of diseases (Yang and Wang, 1993; Mukhtar and Ahmad, 2000). A green tea (non-fermented) produced from the leaves of Camellia sinensis evergreen plants and contains more catechin than black tea and oolong tea. The green tea polyphenolic compound having catechins about 10% of dry weight, including epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin (EC), and epicatechin gallate (ECG) where EGCG accounts for just about 50% of total catechins (Balentine et al., 1997). Different studies have shown that the catechin extracted from Camellia sinensis have following bioactivities and pharmacological actions including antioxidative (Valcic et al., 1999), anti-inflammatory and immunostimulatory activity (Rahman et al., 2006), antiarthritis (Haqqi et al., 1999), antiangiogenic (Sartippour et al., 2002), neuroprotective (Weinreb et al., 2004), cholesterol-lowering (Raederstorff et al., 2003), prevention of cancer and cardiovascular diseases (Kavanagh et al., 2001), antibacterial (Gradisar et al., 2007) and antiviral effects (Weber et al., 2003; Oh et al., 2013).
GTP’s has antiviral activity against different viruses (He et al., 2011) and prevent replication of HIV in peripheral blood cells (Fassina et al., 2002). Influenza virus infectivity was amended with catechins not only by precise interaction with viral hemagglutinin but also viral RNA synthesis in cells (Song et al., 2005). In addition, galloyl group present in catechins play a major role in viral RNA polymerase which inhibit the endonuclease activity of viral RNA polymerase against influenza virus (Kuzuhara et al., 2009). Anti-HCV activity of EGCG by inhibiting viral entry and green tea catechins concurrently inhibit the viral reproduction, inflammation and virus induce carcinogenesis which may be helpful for prevention and treatment of chronic HCV infection (Lin et al., 2013; Ciesek et al., 2011).

Green tea and EGCG inhibit the adenovirus at several stages of replication that directly inactivate the virus particles, inhibiton intracellular virus growth and viral protease, adenain (Weber et al., 2003). EGCG has in vitro antiviral effect by the deformation of phospholipids which inactivate the virus and interfering with reverse transcriptase and protease activity, blocking gp 120-CD4 interaction by binding to CD4 and inactivating virions of HIV (Liu, et al., 2005; Yamaguchi et al., 2002).

The main reason of all poultry vaccination is to provoke protective immune response(s) against field virus/or diseases to decrease or avert economic losses (Haygreen et al., 2005). Conversely, from time to time vaccination failure encountered because vaccination on poultry flocks does not confer protection against viral diseases. The accurate cause of these cases of so-called ‘immunity breakdown’ is frequently ambiguous. While poor vaccine quality is one of numerous potential factors that could lead to vaccination failures, poultry farmers frequently blame vaccines for vaccination failures and related economic losses (Chaudhry and Chaudhry, 1996). That is way antiviral drugs that are capable of preventing an infection or stopping it once started are important as a second arm of antiviral defense.
The present study was design for

- Screening of the antiviral compounds for use in commercial poultry against fowl adenovirus type 4 (FAdV-4) in broiler chicks.
- Propagation of FAdV-4 was on Vero cell line.
- Cytotoxicity of green extract and its isolated Polyphenols were performed on Vero cells for initial screening for use in in-vitro and in-vivo antiviral activity.
- In-vitro antiviral activity of green tea extract and its Polyphenolic compounds were determined in cell culture system.
- Antiviral activities of these tested compounds in vitro were evaluated and in-vivo in broiler birds to adjust its dose for use in poultry.
- Calculation of selective index (SI) which was the ratio of 50% cellular toxicity (CC$_{50}$) over 50% effective concentrations (EC$_{50}$).
CHAPTER: 2

REVIEW OF LITERATURE

Tea:

Origin and nature of tea:

Tea instigated in China, perhaps long ago as 2700 BC (Taylor et al., 2005). Tea is one of the most popular beverages in the world because of its health benefits for human consumption. Tea a product made from an evergreen tree or shrub from Theaceae family, species *Camellia*. Tea is usually produced from the leaves of two varieties include: (*Camellia sinensis*) Chinese tea’s shrub and Indian tea tree (*Camelia assamica*) (Gramza et al., 2005). Tea is grown in more than 30 countries (Graham, 1992) while China, Japan, Taiwan, India, Bangladesh, Sri Lanka and some central African countries like Kenya are the major producers (Shaheen et al., 2006). Of the tea produced globally, 78% is the black tea frequently consumed in Western states and a few Asian states; green tea is 20% of the total tea produce, which is usually consumed in Asia, Middle East and some parts of North America. Remaining 2% oolong tea, this is mostly produced in southern China (Kanwar et al., 2012). Internationally per capita consumption of tea is 40 L per year (Vinson et al., 2004), around 3 million metric tons of tea is produced yearly, increasing at rate of 2.1% (Yang and Landau, 2000). On the basis of the processing procedures, it can be usually divided into green tea (non-fermented) formed by steaming and drying the fresh leaves to inactivate the polyphenol oxidase and therefore prevent further oxidation, oolong tea (semi-fermented) produced as the fresh leaves are submitted to incomplete fermentation before drying, black tea (fermented) passing through a phase of post-harvest fermentation before drying and red (*Pu-Erh*) teas is attained by microbes (Gao et al., 2008). In recent times, green tea beneficial effects on health have fascinated a lot of interest among scientists and the general public (Dube et al., 2010).
Green Tea:

Green tea is prepared from the unfermented leaves of the plant *Camellia sinensis*. It is this minimal treatment, which lead to preserve a high proportion of active polyphenols in comparison with oolong (partially fermented) or black (fully fermented) tea (Chopade *et al.*, 2008; Nagaya *et al.*, 2007). Conventionally, green tea in meticulous has been addicted in order to remove toxins such as alcohol, alleviate pain, improve flow of blood and resist disease (Kao *et al.*, 2006). Catechin in green tea contains several isomers, including EGCG, EC, ECG, EGC and catechin, EGCG with being a major component (Yamaguchi *et al.* 2002). These compounds have radical scavenging actions (Chen *et al.* 2002), antimutagenic activities (Yen and Chen 1995), anti-oxidative activities (Yokozawa *et al.* 2000) and a wide range of antimicrobial activities against bacteria (Matsumoto *et al.*, 2012; Araghizadeh *et al.*, 2013), fungi (Hirasawa and Takada, 2004) and viruses (Lin *et al.*, 2013). The flavonoids present in green tea polyphenol having two aromatic rings A and B with hydroxyl groups (Hara, 2011). The active hydrogens of hydroxyl group in the molecular structure of GTP’s may terminate chain reaction of excessive free radicals, otherwise result in pathological changes in the human body. Tea polyphenols may increase the activity of glutathione peroxidase and superoxide dismutase (SOD) and the scavenging rate is much stronger than vitamins C and E (Sharangi, 2009).

Chemistry of green tea:

The green tea chemical composition is composite and partly defined. The chemical component of tea leaves are polyphenols (catechins and flavonoides), alkaloids (caffeine, theobromine, theophylline, etc.), volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), inorganic elements (e.g., manganese, aluminium and fluorine), etc. Green tea contains six primary catechin compounds namely catechin, gallocatechin (GC), EC, EGC, ECG and EGCG which later become the most active component. The content of green tea and black tea polyphenols differed from 30% to 40% and
3% to 10%, correspondingly (Sharagni, 2009). The most abundant elements in green tea are polyphenols, especially flavonoids such as the catechins, catechin gallates and proanthocyanidins. The fresh leaves contain caffeine (approximately 3.5% of the total dry weight, or about 50 mg/cup when brewed), theobromine (0.15–0.2%), theophylline (0.02–0.04%) and other methylxanthines, lignin (6.5%), organic acids (1.5%), chlorophyll (0.5%) and free amino acids (1–5.5%), in addition to the unique amino acid theanine (4%) and many flavour compounds are also present in much smaller amounts (Graham 1992: Sabu et al., 2010). EGCG is the most abundant of these, containing about 50% of the catechin pool; EGC is about 20%, ECG 13% and EC 6% (Hara 2001).

**Antioxidant potential of green tea Polyphenols (GTP's):**

Oxidative stress happens when there is disparity involving prooxidant relative to antioxidant and defense mechanism relevant to antioxidant. There is increased production of reactive oxygen species (ROS) and impaired antioxidant defense (Shah and Channon, 2004). The prospective health benefits of GTP’s have been partly attributed to their antioxidative property (Mukhtar and Ahmad, 2000). The green tea consumption with balanced controlled diet improves total antioxidative status, which protects humans against oxidative damage (Erba et al., 2005). The GTP’s B ring having trihydroxy and vicinal dihydroxy groups is the favored site for antioxidation (Yang et al., 2002). Chain autoxidation break to give hydrogen atom from Phenolic hydroxyl group with the creation of stable free radical which blocks additional oxidation processes (Xi et al., 2009). GTP’s avoid the configuration of ROS and chelate the metal ion strongly (Kanwar et al., 2012). The action of enzymes for the production of ROS (protein kinase and xanthine oxidase) is also blocked by GTP’s (Quideau et al., 2011). Beside ROS scavenging activity of GTP’s, it detoxifies phase 11 conjugating enzymes (glutathione S-transferase, sulphotransferase, glucuronidase) (Higdon and Frei, 2003). Tea catechins could slow the oxidative damage to Hela cells by 12-O-tetradecanoylphorbol-13-acetate (TPA), averted
Cu²⁺ mediated oxidation of low density lipoprotein (LDL) and blocked ROS production of NADPH-cytochrome P450-mediated oxidation (Surh, 1999). The reactive Carbonyl species (RCS) such as glyoxal (GO), 3-deoxygluosoone (3-DG) and methylglyoxal (MG) readily transform arginine, lysine and cystein residues of protein appearance of advance glycation end products (AGEs) which are related with age-related and chronic diseases (Wang and Ho, 2009). The RCS generated from Maillard reaction which can actively trap by GTPs (Lo et al., 2006).

**Anti-cancer and anti-metastatic activities:**

Tea polyphenols act as powerful antioxidants that play an important role in the prevention of cancer by reducing deoxyribonucleic acid (DNA) damage in the cell activation leading to cancer malignancy (Vasisht et al., 2003). The tea polyphenols intracellular bio-antimutagensis is achieved by modulation of pro-mutagens, repression or blocking and modulation of DNA (Gramza et al., 2005). Signal transduction pathways play a role for intervening biological processes and sustaining cellular homeostasis. Thus modifications of these pathways lead to different types of diseases such as cancer. GTP’s prevent cancer in the regulation of signal transduction pathways by altering the expression of genes that are implicated in cell proliferation, angiogenesis and apoptosis (Kamwar et al., 2012). The basement membrane type IV collagen degradation lead metastatic progression of cancer, green tea catechins having inhibitory effects on collagenases or matrix metalloperoteinases (MMPs) include MMP-2, MMP-9 and MMP-3 (stromelysin) derived from cancer cells (Isemura et al., 2000). The green tea anti-tumor mechanism emerges to include the initiation of apoptosis by the production of H₂O₂ (Yang et al., 2000), NF-κB inhibition (Fujiki and Suganuma, 2002), restrain cell cycle progression (Ahmad et al., 2000), activation of the mitogen-activated protein kinase cascade (Saeki et al., 2002) and attach to a 67kDa laminin receptor (Tachibana, 2011). Green tea induces apoptosis in cancer cells by a mechanism including cell cycle arrest due the presence of high molecular fraction (Ohata et al., 2005). EGCG
in nasopharyngeal carcinoma cells and B-cell lymphoma models inhibits the Epstein-Barr virus (EBV) spontaneous lytic replication and act as anticancer and chemopreventive agents against EBV-associated malignancies (Liu et al., 2013).

**Reduction in Cardiovascular disease (CVD):**

There are no of risk factors for the development of CVD including diastolic blood pressure, systolic blood pressure (SBP) and endothelial function (Johnson et al., 2012). The CVD risk is increase with high level of cholesterol. There are various causative factors including stress, regular utilization of animal fat or intake of refined sugar or oil. Green tea has useful for cardiovascular system by lowering cholesterol and averting platelet clumping (Sharangi, 2009). The extract of green tea high in catechins leads to diminution SBP, body fat and low-density lipoprotein (LDL), which contributes to a reduce in CVD risk and obesity in human beings (Nagao et al., 2007). In the arterial wall LDL deposit, this subjected to oxidation. Afterward, oxidation of LDL induces alteration in lipoproteins, motivates inflammatory reactions, and causes monocytes and monocytes-derived macrophages to amass in great amount of oxidized LDL and form atherosclerotic plaques and lipid-laden foam cells. Principal means for the valuable outcome tea include antioxidative, vasculoprotective, anti-inflammatory, antithrombogenic and lipid lowering properties of tea polyphenols (Stangl et al., 2006). The total cholesterol content and triglycerides in blood reduce due green tea leaves in diet of rat, with the enhancement of liver enzyme SOD and phase 11 activities (Khan and Mukhtar, 2007). Green tea implicated cellular protection against ROS by enhances the activity of enzymes SOD in serum and catalase expression in aorta (Negishii et al., 2004). Green tea catechins reduce the absorption of cholesterol and triglycerides that it enhances the excretion of fat. Their intakes avert the emergence of atherosclerotic plaque by affecting the lipid metabolism through diverse pathways (Raederstorff et al., 2003). The control of obesity and hypertension by the use of tea may also reduce the occurrence of CVD.
Pathogenesis of atherosclerosis and coronary circulation relate to the impaired endothelium-derive nitric oxide activity linked with future CVD events. Oxidative stress was increased with endothelial dysfunction and reversed by antioxidants in green tea (McKay et al., 2002). Green tea inactive the products through facilitating the metabolism of chemical carcinogens that readily excreted, suggested that its anticacinogenic effects by increased glucuronidation by UDP-glucuronosyl transferase induction

**Other beneficial effects of Green tea:**

The most diseases linked to the initiation of pro-inflammatory genes by tumor necrosis factor (TNF) (Kundu and Surh, 2008). The expression of inflammatory gene such as Cyclooxygenase-2 (COX-2), inflammatory cytokines and inducible nitric oxide synthase (iNOS) when TNF exposed to nuclear factor Kappa-light-enhancer activated B cells (NF-κB) activated in most cells types. Catechins of green tea inhibit iNOS and COX-2 expression by blocking activation of NF-κB and increase the production of anti-inflammatory cytokines IL-10 (Wang et al., 2011). Tea catechins altered the expression of Thi1/Th2 cytokines which concealed myocardial cell infiltration and fibrosis in induced autoimmune myocarditis in rat (Suzuki et al., 2007). Green tea and green tea extract beneficially modify the glucose metabolism in type 11 diabetes mellitus by increases tyrosine phosphorylation of insulin receptor and reduce gene expression of the gluconeogenic enzyme (Wu et al., 2004). The imbalance between energy intake and expenditure of energy lead to a metabolic disorder known as obesity. Green tea catechins down-regulate the gene expression of fatty acid synthase in the nucleus and stimulating energy expenditure of the mitochondria in the cell observed in cancer cells to growth inhibition or suppression of lipogenesis preventing weight gain (Rudelle et al., 2007). Neurodegenerative diseases such as Alzheimer’s and Parkinson are produced by oxidative stress can be protected by catechins (Zaveri, 2006; Pan et al., 2010). GTP’s and GTE have positive effects on the activity and proliferation of the bone cells which allied with bone mineral density increase (Muraki et al.,
Liver fibrosis by galactosamine induced hepatic injury was prevented by green tea catechins through collagens gene expression down regulation (Abe et al., 2007). Recently, epidemiological study shows that lower prevalence of cognitive impairment in human associated with the consumption of green tea (Suzuki et al., 2012).

**Anti-infective properties of Green tea:**

**Anti-bacterial and fungal activity:**

Plant-derived anti-microbial compounds have weak activity contrast to ordinary antibiotics. Though, drug resistance, imperfect specificity and unavoidable side effects limit the efficiency of antibiotics (Sharma et al., 2012). The polyphenol stuffing of green tea have been account to inhibit no of pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus*, *helicobacter pylori*, *Streptococcus sobrinus*, *Streptococcus mutans*, *Shigella dysentery*, *Shigella flexneri* and *Salmonella typhi* (Taylor et al., 2005). There is several mechanism of antibacterial action of *Camellia sinensis*. One of the mechanisms is that *Camellia sinensis* permanently damages the bacterial cytoplasmic membrane, which can alter the adhesion of bacteria to the cells (Lee et al., 2009). Other proposed mechanisms of *Camellia sinensis* embrace upsetting the activity of dihydrofolate reductase (DHFR), pathogens require this enzyme for the synthesis of purines and pyrimidines (Chung et al., 2003). Green tea catechins inhibit bacterial DNA gyrase that bind to ATP binding site of the gyrase B subunit (Gradisar et al., 2007). EGCG effectively inhibiting protein tyrosine phosphatase in *Proventa intermedia* (Okamoto et al., 2003).

Green tea methanol: water extract inhibit urease enzyme of *Helicobacter pylori* which is necessary for its colonization (Shoae Hassani et al., 2009). Green tea extract has been revealing to avert gastric mucosal inflammation, cell proliferation and apoptosis stimulated by *Helicobacter pylori* infection in mice (Stoicov et al., 2009; Akai et al., 2007). The ROS generated from catechins have antibacterial effects (Arakawa et al., 2004). Dental caries and periodontal diseases caused by endogenous oral flora. The major etiological agents of
dental caries are *Streptococcus sobrinus* and *Streptococcus mutants*. The toxic end metabolites such as protein tyrosine phosphatase, alkaline phosphatase, collagenase, and gingipains of periodontopathic bacteria neutralize by EGCG, these end products lead to destruction of gingival tissues and development of periodontitis (Okamoto *et al*., 2003). Gram-negative bacteria are more resistant to green tea catechins than Gram-positive bacteria due to presence of negatively charged liposaccharide in cell wall (Gramza *et al*., 2005). Green tea catechins also encompass discriminatory immunomodulatory property on pathogens, as was observed for *Legionella pneumophila* (Matsunaga *et al*., 2001). EGCG was established to restrain the growth of *L. pneumophila* in macrophage due to modification in immune response of macrophages and increased production of pro-inflammatory cytokines (Steinmann *et al*., 2013).

EGCG has antifungal activity against *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Candida albicans* and *Cryptococcus neoformans* were first analysed in 1991 (Okubo *et al*., 1991). EGCG cause lysis of the canidia and hyphae of *Trichophyton mentagrophytes* which suggesting its antidermatophytic effects. EGC, ECG and EGCG cause metabolic instability of *C. albicans*. Of this EGCG Inhibit *C. albicans* proteasomal chymotrypsin-like activity *in-vivo* leads to the cellular metabolic and structural disruption of the yeast (Evensen and Braun, 2009).

**Antiviral activity of green tea:**

**Effect against HIV:**

Acquired immune deficiency syndrome (AIDS) caused by Lentivirus of the family *Retroviridae*. An estimated 33 million people are infected with HIV worldwide. HIV/AIDS represent a most important cause of morbidity in developing countries (Simon *et al*., 2006). Globally, HIV/AIDS is a major public health crisis due to lack of effectual treatment regimens. The CD4+ T-lymphocytes was the identified retroviral receptor and HIV infects CD4+ T-lymphocytes, follow-on their depletion, which leads to immunodeficiency (Bour *et al*., 1995). EGCG directly bind to D1 domain of CD4+ which inhibit the
binding of gp 120 at physiological concentration (Williamson et al., 2006). EGCG act as an allosteric reverse transcriptase enzyme inhibitor of HIV-1 result in a decrease in p24 antigen concentration (Li et al., 2011). EGCG has ability to inhibit the HIV-1 integrase protein, which is liable to insert the HIV proviral DNA into the genome of infected cells (Jiang et al., 2010). The galloyl motety present in catechins have the ability to decrease the binding between the integrase and the viral DNA as a result reduction in HIV-1 integration take place. Tea catechins mediated partly by inhibiting HIV bind to target cell surface and virus inactivation by phospholipids deformation (Yamaguchi et al., 2002). EGCG and its allied compounds have potential to inactivate the infectivity-enhancing ability of human semen (Haubera et al., 2009). EGCG considered as a valuable potential for the treatment of HIV infection in future.

**Effect against Hepatitis C virus (HCV):**

HCV is a serious health hazard globally. HCV is the most important source of chronic liver disease and leading indication of transplantation of liver globally, which infects chronically 160 million individuals (Lavanchy, 2011). Recently, three independent groups search for new antiviral molecules have recognized that EGCG has potential to inhibit the HCV pathway (Ciesek et al., 2011; Calland et al., 2012; Chen et al., 2012). EGCG was shown to increase the formation of lipid-droplet and affect the secretion of very-low-density lipoprotein in hepatocytes (Li et al., 2006). Thus link between lipid metabolism and HCV life cycle, this molecule blocks the early step of the entry process, the attachment step, probably by targeting HCV particle (Galland et al., 2012) and inhibit viral NS3/2A protease or NS5B polymerase (Zuo et al., 2009; Roh and Jo, 2011). The green tea molecules has the capacity to clear HCV from cell culture at 50µM of EGCG in human cells, there was no detectable infectious virus in supernatant after three passages (Galland et al., 2012) and at the same concentration, virus has been clear after two passages (Chen et al., 2012).
Against others viruses:

HBV infection is the most vital health issue publically throughout the world. Approximately 40% of worldwide human population has contact with HBV which is transmitted by perinatally, sexually and parentally (Shepard et al., 2006). HBV messanger ribonucleic acid (mRNA) analysis revealed that green tea extract impede the transcription of HBV and the diminution of HBV mRNA is the major key function of GTE anti-HBV activity (Xu et al., 2008). HBV chronicity promotes by HBeAg an immune regulatory protein which involved in immune tolerance (Chen et al., 2005). Green tea extract during HBV infection overcome immune tolerance of the host. EGCG significantly inhibit the replicative intermediates of RNA synthesis that reduce the production of covantly closed circular deoxyriboonucleic acid (cccDNA) (He et al., 2011).

Norovirus belong to the family Caliciviridae is one of the most significant food-borne pathogens in the globe which cause epidemic gastroenteritis in humans (Leuenberger et al., 2007). Green tea catechins may be a proficient contact food sanitizer which is included to food having antiviral activity against noroviruses (Oh et al., 2013). Adenovirus causing gastroenteritis, conjunctivitis in human and FAdVs are ubiquitous in poultry in the genus Aviadenovirus under the family Adenoviridae which causes many disease conditions in chickens including IBH-HPS, respiratory infections (Balamurugan et al., 2002). Green tea catechins and infusions reduced infection caused by adenovirus and adenain a viral protein in Human hepatocellular liver carcinoma (HepG2) cells is major target in preventing infection (Weber et al., 2003). Influenza A and B viruses are a most important cause of respiratory disease in human particularly Influenza A virus have the capacity to re-emerge every 10-50 years (Garcia-Sastre, 2011). Influenza virus infectivity in cell culture is affected by green tea which prevents virus absorption to Madin-Darby canine kidney (MDCK) cells through virus agglutination (Nakayama et al., 1993). Acidification of intracellular compartments such as endosomes and lysosomes was inhibited by green tea catechins which results in influenza growth inhibition in cell
culture (Imanishi et al., 2002). A statistically significant protective effect on infection caused by clinically defined influenza virus was observed in a randomized trial (Matsumoto et al., 2012). The change in viral membrane properties of influenza virus added to the antiviral activity of green tea catechins (Song et al., 2005). There was no specific treatment of Influenza in poultry green tea by products has great potential to prevent viral infection and spread in epidemic area in animals at very reasonable price (Lee et al., 2012). A viral disease known as herpes simplex caused herpes simplex type 1 and 2 (HSV-1 and 2). Worldwide distribution of herpes simplex virus infection is amid 65 and 95% (Chayavichitsilp et al., 2009). EGCG directly damage the virion which decreases more than 1000-fold titres of HSV-2 in 10-20 min and at same level in 30-40 against HSV-1 titres (Isaacs et al., 2008). In-vivo, between pH 4.0 and 6.6 dimers of EGCG more effectively inactivates the HSV-1 and HSV-2 than monomer of EGCG which is more potential for decreasing the spread HSV (Issacs et al., 2011). The infectious mononucleosis which is intimately associated with T-cell lymphoma, nasopharyngeal carcinoma, Burkitt’s lymphoma and Hodgkin’s disease caused by human herpesvirus known as EBV (Bravender, 2010). EGCG decrease the expression of EBV lytic proteins (Rta, EA-D and Zta) by reducing the transcription of immediate-early genes (Chang et al., 2003). The causative agent of hand, foot and mouth disease is enterovirus 71. A single stranded ribonucleic acid (RNA) virus causes different clinical appearance, including cutaneous visceral and neurological diseases. Replication of enterovirus 71 and infectious virus progeny was inhibited by EGCG (Ho et al., 2009). The antiviral and antioxidant effects of EGCG were positive correlations which suppress replication of virus through modifying the cellular redox milieu.
Adenoviruses (AdVs):

General properties of AdVs:

AdVs are pervasive infectious agents in humans, poultry, and wildlife worldwide (Russell, 2000; Fitzgerald, 2008). AdVs are able to infect a wide range of species or a variety of actively dividing as well as post-mitotic cells. However, those AdVs that produce disease cause lesions in a narrow range of organs (Russell, 2000). Generally, they exhibit a low level of virulence (Schrenzel et al., 2005). Most of the AdVs are species-specific (Wold and Horwitz, 2007), and the host range of any AdV is restricted to one or, at most, a few closely-related species of animals (Sambrook et al., 1980). Most of the knowledge on adenoviral structure, biology, genomic organization and replication originates from studies done with human adenoviruses (HAdVs) (Benkő et al., 2005). The HAdVs have been recognized as significant viral pathogens, with higher morbidity and mortality among immunocompromized people (Echavarria, 2008). The presence of predisposing factors such as compromised immune status, concurrent infection, stress; and mixing of a carrier population with immunologically naïve human, livestock or poultry species is required for the development of severe or fatal disease (Fitzgerald, 2008). Pathology is caused by the process of virus replication and lysis of susceptible cells (Adair and Fitzgerald, 2008). An AdV was first isolated from a dog with infectious canine hepatitis (Rubarth, 1947), and the intranuclear inclusions detected in hepatocytes were thought to be due to a filterable agent (Cowdry and Scott, 1930). The first HAdV was accidentally isolated from human adenoids (Rowe et al., 1953) and later named as “adenovirus” (Enders et al., 1956). The first avian AdV to be identified was from a respiratory disease outbreak in bobwhite quail (Colinus virginianus) (Olson, 1951).

Taxonomy and classification of AdVs:

Members of the family Adenoviridae are non-enveloped, 70-80 nm in diameter, single linear, double-stranded deoxyribonucleic acid (dsDNA) viruses
which have a characteristic icosahedral capsid of 240 non-vertex capsomeres (hexons), and 12 vertex capsomeres (pentons) each with one or two fibers protruding from the penton base (Russell, 2009). Although all AdVs share a common structure, HAdV-40 and HAdV-41 (Russell and Benkő, 1999) and viruses of Aviadeniovirus (Adair and Fitzgerald, 2008) possess two fibers that project from the penton base (Gelderblom and Maichle-Lauppe, 1982). The size of the genome varies from 26-45 kbp with inverted terminal repeats linking the genome to the terminal protein at each end (Benkő et al., 2005). While structural divergence between genera exists but the fundamental feature in all the genera of AdVs are maintained (Russell, 2009). Throughout the AdVs family, there is well conserved central part of the genome whereas wide variation at two ends in length and content (Benkő et al., 2005).

The family Adenoviridae is designated by the International Committee on Taxonomy of Viruses (ICTV) to contain four genera: Mastadenovirus, Atadenovirus, Aviadenovirus, and Siadenovirus based on genomic organization, virion properties, structure, size and serological differences (Benkő et al., 2005). A fifth, distinct genus containing fish and snake AdV has been proposed (Benkő et al., 2002). The AdVs that infect birds have been classified using the highly conserved group-specific epitopes of the hexon protein (van Regenmortel et al., 1997), and designated as group I, II or III avian adenovirus based on their common group antigen (Ag) (McFerran and Smyth, 2000; Fitzgerald, 2008; Pierson and Fitzgerald, 2008). Presently, the genera of the family Adenoviridae are classified as: Mastadenovirus mammalian AdV, Aviadenovirus group I avian AdV, Siadenovirus group II avian AdV, and Atadenovirus group III avian AdV (Fitzgerald, 2008).
**Aviadenovirus:**

*Aviadenovirus* include group I avian AdV of chicken, turkey, duck and goose (Adair and Fitzgerald, 2008). A subgroup of *Aviadenovirus*, commonly referred to as FAdV consists of five species, designated as A-E based on restriction enzyme analysis (REA) of viral DNA (Zsak and Kisary, 1984). On the basis of partial cross neutralization and phylogenetic difference of hexon protein L1 loop, FAdV further delineated into 12 serotypes, FAdV-1 through FAdV-11 with FAdV-8 subdivided into FAdV-8a and FAdV-8b (Benkő et al., 2005).

*Aviadenovirus* are diverse from other genera by the existence of two fibers per vertex (Gelderblom and Maichle-Lauppe, 1982), and only infect birds (Benkő et al., 2005).

The ratio of homologous: heterologous titer in both direction that is greater than 16 described as one serotype of FAdV, that showing no cross-neutralization with others (Benkő et al., 2005). If the titer is between 8-16, serotypes can be differentiated by biophysical or biochemical methods (Hess et
Classification of 12 FAdV reference strains based on real-time polymerase chain reaction (PCR) and consequent high-resolution melting point-curve analysis of three regions of the hexon gene has been developed (Steer et al., 2009). The FAdV’s share a common group Ag with viruses isolated from geese, ducks and turkey (Adair and Fitzgerald, 2008), but demonstrate a broad antigenic variation causing a variety of diseases among diverse poultry species (Hess, 2000). Most FAdVs grow well in these cells with characteristic cytopathic effects (CPE) of cell rounding, refractility, and surface detachment (Adair and McFerran, 2008).

**FAdV-4:**

*Aviadenoviruses* are responsible for IBH-HPS, respiratory disease, drop in production or feed conversion, nectrotizing pancreatitis, and adenoviral erosion. The anemia and severe depletion of lymphoid from spleen and bursa Fabricius are also associated with *aviadenoviruses* (Saifuddin and Wilks, 1992). Group 1 *aviadenovirus* are commonly referred as fowl adenovirus (FAdV) having five species and twelve serotypes. FAdV-4 belong to FAdV C is solely responsible for IBH-HPS (Adair and McFerran, 2008).

**IBH-HPS:**

IBH-HPS is an emerging, economically important disease of 3-6 week old broiler chickens (Balamurugan and Kataria, 2004). IBH-HPS has been occasionally reported in broiler breeder pullets aged between 2-32 weeks (Asrani et al., 1997) and in pigeons (Naeem and Akram, 1995). The disease was first reported in 1987 (Anjum et al., 1989) and later in India, Iraq, Russia, Japan, Mexico, and several Central and South American countries (Abdul-Aziz and Hasan, 1995; Ganesh and Raghavan, 2000). It is characterized by a sudden onset of mortality (20-80%), hydropericardium and friable, swollen livers with necrosis (Balamurugan and Kataria, 2004). As IBH-HPS shares the liver lesions and presence of INIB in hepatocytes of broiler chickens with IBH, the disease is also called IBH-HPS, infectious hydropericardium, hydropericardium hepatitis syndrome or “Angara disease” (Adair and
Fitzgerald, 2008). Severe hydropericardium and high mortality distinguishes IBH-HPS from IBH (Balamurugan and Kataria, 2004). Epidemiological investigations have shown FAdV-4 as the causative agent (Voss et al., 1996; Mazaheri et al., 1998) with a genomic similarity of the viruses involved in IBH-HPS outbreaks in different geographical areas (Hess et al., 1999).

The transmission of IBH-HPS occurs horizontally through contaminated feces and fomites; and the disease in progeny occurs through vertical transmission (Akhtar, 1994; Mazaheri et al., 2003). Several studies have been successful in experimental reproduction of IBH-HPS in broiler chickens (up to 3 weeks of age) by inoculation of liver homogenate (LH) of HPS/IBH from outbreaks (Ganesh, 1998) or purified virus prepared from field isolates (Ganesh et al., 2001). Pathogenicity studies with IBH-HPS have suggested that a synergism with chicken infectious anaemia virus (CIAV) or prior immunosuppression is required for experimental reproduction (Toro et al., 1999; Toro et al., 2000). An association with CIAV is also shown to be necessary for vertical transmission of FAdV associated with IBH-HPS (Toro et al., 2000), however, vertical transmission and occurrence of IBH-HPS in progeny has been demonstrated in specific pathogen free (SPF) chickens (Mazaheri et al., 2003).

Transmission of FAdV infections in chickens:

The FAdV are known to be commonly transmitted both vertically and horizontally from viremic parent flocks to the progeny (McFerran and Adair, 1977). Vertical transmission is the most likely the major process of spread of FAdV between chicken flocks, but horizontal transmission within a flock occurs readily as the virus is present in all excretions and in high numbers in feces (Adair and Fitzgerald, 2008). With vertical and horizontal transmission, and the ability to persist in the environment, FAdVs have evolved as successful pathogens worldwide (Adair and Fitzgerald, 2008). Vertical transmission of FAdV was first observed in chicken embryos (Yates and Fry, 1957). FAdVs have also been detected in eggs as a contaminant (Cook, 1968). The FAdVs have
been isolated from embyronating eggs of SPF chickens inoculated 24 with the virus (Mazaheri et al., 2003). Vertical transmission has also been demonstrated by detection of FAdVs DNA in day-old chicks (Grgic et al., 2006). Vertical transmission of FAdVs appears to correlate with the neutralizing Ab in the blood (Dawson et al., 1981). Similarly, development of neutralizing Ab appears to coincide with virus excretion (Adair and Fitzgerald, 2008). Others have observed that vertical transmission is prevented in the presence of VN Ab or strong immune response to FAdVs (Philippe et al., 2007). A strain or serotype specific variation in vertical transmission of FAdVs has also been reported (Dawson et al., 1981; Mazaheri et al., 2003). As the level of maternal Ab in broiler chickens wane at around 3 weeks of age there would be opportunity for suitable strains to spread from the intestine and cause disease (Adair and Fitzgerald, 2008). Vertical transmission of FAdV associated with IBH has been found in the egg, embryonating egg or newly hatched chicks (Reece et al., 1985; Saifuddin and Wilks, 1991). Fomites, people and vehicles also can be important modes of spread of FAdVs (Adair and Fitzgerald, 2008). Though, very little is recognized about the activities of the FAdVs in large commercial flocks (Grimes, 2007).

**Other diseases caused by avian AdVs:**

There are several other important diseases affecting poultry which are caused by viruses belonging to the genera *Siadenovirus* and *Atadenovirus* of the family *Adenoviridae*. Viruses in the genus *Siadenovirus* typically produce disease without immunosuppressive factors, virus from the genus *Atadenovirus* cause egg drop syndrome (Adair and Symth, 2008). Natural outbreaks of gizzard erosions in broilers are characterized by mortality in young broilers without clinical signs of infection (Manarolla et al., 2009). On gross examination, distended gizzards with haemorrhagic fluid and multiple black patchy erosions are found (Abe et al., 2001).
Morphology of FAdV’s:

The icosahedral adenoviral capsid structure is composed of three major structural proteins: hexon, fiber, and penton (Crawford-Miksza and Schnurr, 1996). The hexon is the most abundant structural protein on which group, type and subgroup specific antigenic determinants as well as neutralizing epitopes are located (McFerran, 1981; Russell, 2009). There are two functional components in the hexon having the conversed pedestal regions P1 and P2 and the L1-L2 a variable loops (Athappilly et al., 1994). The L1 loop contain the highest variability among FAdV serotypes and antigenic determinants, and other loop regions, except L3, other loop regions are situated on the surface of the hexon protein and interact with host immune responses (Raue and Hess, 1998).

The fiber of FAdV is divided into three sections: the N-terminus which binds to the immunoglobulin (Ig) domain similar to D1 of Coxsackie and adenovirus receptor (CAR) of HAdV (Tamanini et al., 2006); the middle region; and the C-terminus which makes up the knob. Although two fiber molecules are encoded by CELO, the long fiber is responsible for a CAR-dependant infection pathway (Tan et al., 2001).

The FAdVs are estimated to compose 80-7% protein, mostly derived from hexons and pentons of the capsid (Laver et al., 1971). The genome of FAdV is composed of 41-45 kbp linear, dsDNA condensed within the virion by virus-encoded core proteins (Laver et al., 1971). It replicates unidirectionally by viral DNA polymerase that copies a single strand at a time (Laver et al., 1971). Viral transcription utilizes host cellular RNA polymerase II or III with DNA replication and virus assembly in the nucleus, and the virus is released upon cell rupture (Wold and Horwitz, 2007). During productive infection most AdV induce the synthesis of soluble, complement-fixing Ag of the hexon protein (Sambrook et al., 1980), and it may be associated with cross-reactivity with other members in the same group or other groups (Norrby and Wadell, 1969).
Targeting the liver leading to infection of hepatocytes occurs with HAdV-5 by attachment of hexon to coagulation factor X (Waddington et al., 2008).

**Genome:**

The mastadenovirus genome is around 36 kilobase (kb) and consists of four early regions (E1 to E4), and five late regions (L1 to L5), while the FAdV genome is around 45 kb and is the largest genome among members of the family *Adenoviridae* (Chiocca et al., 1996; Ojkic and Nagy, 2000). So far, the complete nucleotide sequences of the genomes of FAdV-1, FAdV-9, FAdV-8, FAdV-4, and turkey adenovirus type 1 (TAdV-1) are determined (Ojkic and Nagy, 2000; Grgic et al., 2011; Griffin and Nagy, 2011; Kajan et al., 2010). The nucleotide sequences at the left and right ends of the genomes of FAdVs representing species C (FAdV-4 and FAdV-10), D (FAdV-2), and E (FAdV-8) are also resolute and evaluated to FAdV-1 (*FAdV-A*) and FAdV-9 (*FAdV-B*) (Corredor et al., 2006; Corredor et al., 2008). Nucleotide sequence homology and amino acid sequence identities are the highest between members of the same species group, while different degrees of variations are present among all FAdVs. Moreover, genome of FAdVs has a similar gene arrangement, propose most likely the related gene functions of all studies FAdVs (Corredor et al., 2008). Interestingly, all analyzed FAdV at the right end genomes, homologues to known ORFs such as lipase and Gam-1 are also found (Corredor et al., 2008). GAM-1, encoded by ORF8, between the same species group among viruses is highly conserved (82%–95.7%). In the following text, interesting features related to FAdVs will be pointed out. FAdVs contain large genomes (FAdV-4 45,667 base pair (bp); FAdV-9 45,063 bp; FAdV-8 44,055 bp) (Griffin and Nagy, 2011; Ojkic and Nagy, 2000; Grgic et al., 2011), although genes encoding a few structural proteins such as V and IX, and early regions E1, E3, and E4 described in mastadenoviruses are missing from them. The genomes of atadenoviruses and siadeoviruses are shorter. For example, the egg drop syndrome virus and hemorrhagic enteritis virus are 33.2 and 26.3 kb, respectively (Hess et al., 1997). The middle part of the adenovirus genome have
highest degree of conservation, which encodes the structural proteins, whereas the two ends, containing mainly regulatory early regions, exhibit a larger variation (Davison et al., 2003). In mastadenoviruses, early regions E1A and E1B are present without exception, while in atadenoviruses, a p32K gene is present in the position of the E1A of mastadenoviruses. The p32K gene is unique to atadenoviruses and has been identified in every member of the genus \textit{Atadenovirus} studied so far. In addition, only the atadenoviruses seem to have some degree of homology with the mastadenovirus E1B genes, namely the 55K protein. Protein IX and V, found in mastadenoviruses, are absent in the avi-, at- and siadenoviruses, suggesting that the virions of these adenoviruses are different structurally. The E3 region seems to exist only in the mastadenoviruses and no homologous genes were detected in any other genera. However, the putative E3 region of siadenoviruses has no homology with the E3 region of mastadenoviruses. Furthermore, the right-hand end of the genome harbors an E4 region where the single gene for the 34K protein of mastadenoviruses has its homologue in atadenoviruses only (Benkő et al., 2005). The presence of tandem repeats (TR) at the right end of the genome is reported for several FAdV’s, namely FAdV-9, FAdV-8, FAdV-4, and FAdV-10 (Ojkic and Nagy, 2000; Corredor et al., 2008; Grgic et al., 2011; Griffin and Nagy, 2011). The FADV-9 with TR-2 containing 13 repeats of 135 bp each is dispensable for virus replication \textit{in vitro} and \textit{in vivo} (Ojkic and Nagy, 2000; 2001). The role of these tandem repeats remains mysterious. The GAM-1 protein is unique to FAdVs only and has functional equivalence to the human adenovirus E1A 243R, E1A 289R, and E1B 19 kDa proteins. GAM-1 of FAdV-1 virus is concerned in escalating cellular transcription by blocking pRb and activating E2F-dependent transcription (Lehrmann and Cotten, 1999). Additionally, lipase ORFs detected in the genome of FAdV-D and FAdV-E species have high identities to lipase from pathogenic avian herpesviruses (Corredor et al., 2008), which is important in virus replication (Kamil et al., 2005).
**Proteins:**

The structural proteins 11, 111, 111a, 1V, V1, V111 and 1X, contains by viral capsid. Proteins named as V, V11, X and terminal protein (TP), which interact directly with the viral DNA, from the viral core (Lehmberg et al., 1999). The mature virion polypeptides nominated by Roman numbers from 11 to X11 (Maizel et al., 1968). The most abundant protein in the virion is protein 11 (hexon protein) which defines the type, group and subgroup antigenic determinants (Norrby, 1969). Protein 111 (penton protein) has different functions, including make sure stable fiber penton base interaction and pentamerization to form a penton base (Karayan et al., 1997). The vertices of the icosahedral capsid interact with the penton base of protein 1V called fiber protein. For viral attachment and internalization of the virus into the cell of the host, this fiber protein is directly responsible for this activity of the virus (San Martin and Burnett, 2003). Generally, the structure of the fiber is divided into three discrete regions (Green et al., 1983). The first region is tail containing of N-terminus which is embedded into penton base. The second region nominated as the shaft having several repeating motifs of around 15 amino acids. The end of the shaft located at the C-terminus is called the knob which play necessary role for viral binding to the cell of the host.

Minor capsid components, including proteins IIIa, VI, VIII, and IX complete the capsid structure and primarily act as cement proteins. The location of each of them and their accurate role in the virus life cycle has yet to be fully understood. The location of polypeptide IIIa has been recently defined as a position below the penton base and this protein is associated with others at the vertex, mainly with the hexon and protein V (Saban et al., 2005). The location of polypeptide VI in the virion appears to be inside cavity of every hexon trimer, connecting the bases of two adjacent peripentonal hexons and tethering the capsid to the core region (Silvestry et al., 2009). The endosome acidic environment trigger the conformational changes lead to release of protein VI from the virion and this disruption of membrane promote the transport of
virus to the nucleus (Wiethoff et al., 2005). Moreover, protein VI harbors a PPxY motif involved in reapid microtubule-dependent intracellular movement and infectivity. Inactivation of the PPxY motif leads to post-entry delay with consequence on reduced infectivity and prevent efficient accumulation of the virions at the microtubule organizing center (Wodrich et al., 2010; Maier and Wiethoff, 2010). Polypeptide VIII is situated at the inner surface of the capsid and it is bound among the peripentonal hexons and the rest of the capsid (Stewart et al., 1993). The smallest minor capsid protein, protein IX, is present as a trimer and helps to stabilize the virion, as mutant virions lacking this protein are less stable than the wild type virus (Colby and Shenk, 1981). Proteins V, VII, μ, and terminal protein (TP) directly interact with the linear dsDNA to form the viral core (van Oostrum and Burnett, 1985; Lehmberg et al., 1999). Polypeptide VII is a major core protein with over 800 copies per virion and together with DNA forms compact repeating structures called ‘adenosomes’ (Tate and Philipson, 1979). TP acts as a primer for DNA replication and the virus genome circularization may be facilitated.

The precursor terminal protein (pTP), is cleaved by virus-encoded protease at two sites, creating TP that is indispensable for virus replication (Webster et al., 1997). About 30 adenovirus non-structural proteins have been described (San Martin and Burnett, 2003). They are generally produced in small quantities and the precise role for most of them is still unknown. To date, the structures of two of the non-structural proteins have been described, DNA-binding protein (DBP) and viral protease (Tucker et al., 1994; McGrath et al., 2003). DBP is a multifunctional nuclear phosphoprotein. It is a most important product of early region E2A and is essential for viral DNA replication. The control of early and late transcription also needs DBP. (Ward et al., 1998; van der Vliet et al., 1978). This protein has two domains: a highly phosphorylated N-terminal domain of 173 aa that contains a nuclear localization signal and the C-terminal domain of 356 aa (Morin et al., 1989). The highly conserved and non-phosphorylated C-terminal domain binds DNA and is active in DNA
replication (Linne and Philipson, 1980). DBP binds single-stranded (ss) and dsDNA as well as RNA. DBP binds cooperatively to ssDNA, protecting it against nuclease digestion and destabilizing the double helix during the elongation phase of DNA replication. Binding to dsDNA occurs in a non-cooperative fashion and is less stable (Tsernoglou et al., 1984; Zijderveld and van der Vliet, 1994).

Proteinases play essential roles at various stages of viral replication, including assembly and maturation of virions. AdV’s encode an endopeptidase that is crucial to the assembly of the virus in the nucleus of infected cells (Weber, 1995). Between 10 and 30 molecules of the protease are packaged into each virion. Cotten and Weber (1995) demonstrated that the adenovirus-encoded 23K protease is required for cleavage of virion precursor proteins.

**Adenovirus replication cycle:**

Adenoviruses (AdV’s) entry into cells involves attachment of the fiber knob to the primary receptor. The Coxsackievius and adenovirus receptor (CAR) is the adenovirus receptor for most but not all HAdVs (Bewley et al., 1999). The existence of CAR on target cells consent virus attachment by HAdVs and also severs as a receptor for a number of AdV’s affects animals (Cohen et al., 2002). The main function of the fiber receptor is to conduct the virion in close proximity to the cell surface, allowing interaction with an integrin molecule. The internalization of virus penton base interacts with the secondary receptor. The virus enters the cell by receptor-mediated endocytosis in a clathrin-coated vesicle and is transported to endosomes where acidification results in partial removal of the capsid. The altered virion escapes into the cytoplasm and is transported to the nucleus, where replication occurs (Meier and Greber, 2004).

According to the times, expression of particular viral genes that divide AdV replication cycle into an early (E) and late (L) phase. Soon after infection, activation of early viral transcription units (E1A, E1B, E2A, E2B, E3 and E4), lead to early proteins that function through transactivation of other AdV transcription units, maximum viral replication occur through cell cycle
deregulation or through host’s antiviral immune system modulation (reviewed in (Russell, 2000). The E2 gene products accumulate which provides a set for viral DNA synthesis until the death of host cell after 7 hours of infection. The three proteins, the DNA-binding protein (DBP), the AdV DNA polymerase and the pTP required for DNA replication encoded by genome of AdV. In addition, replication enhance by cellular proteins (i.e. nuclear factor I, II and III) (Mul et al., 1990). Replication starts with a unique protein-priming mechanism in which the primer pTP interacts with the viral DNA polymerase, resulting in a tight preinitiation complex at the origin of replication, situated within inverted terminal repeats (ITRs) of the AdV genome (Brenkman et al., 2002). Then covalently linked to a serine residue in the pTP, a CAT trinucleotide is synthesized from 4–6 nucleotides opposite the template strand. The pTP–CAT intermediate then ‘returning’ to permit pairing with the first three nucleotides of the template, intervene by sequence repeats in the ITRs. The DBP and Ad DNA polymerase required for further elongation. During late stage of infection, pTP cleaved by proteinase of AdV to a smaller TP. When one of the parental strands was amplified displaced single-stranded molecule may then serve as a template for DNA synthesis by creating a panhandle structure by the self-complementary ITRs (Leegwater et al., 1988). After the initiation of DNA replication, major late promoter of AdV activated leads to the expression of late genes. Beginning of viral DNA replication in conjunction with the production of large amount of structural proteins provides the best promising condition for virus assembly. The trimeric hexon capsomeres necessitate the L4 100k protein for the assembly of virus (Hong et al., 2005). Penton capsomeres, having a pentameric unit and trimeric fiber, gradually assemble in the cytoplasm. Following their production, penton and hexon capsomeres are introduced into the nucleus and assembly of virion take place (Schmid and Hearing, 1995). Proteins 1Va2 and 1X are imperative for packaging of full-length genomes into the capsid (Zhang and Imperiale, 2003). Assembled virions in the nucleus of infected cells and about 3 days after infection, Ad
progeny released by cell lysis, caused by the adenovirus death protein (ADP) which expressed at high levels late in infection and endorses the release of virions from infected cells encode by the E3 region (Tollefson et al., 1996).

**Prevention and Control:**

**Biosecurity:**

Appropriate biosecurity measures are important in the control of horizontal spread of Ad’s within a poultry house or farm. This is especially true in the prevention of IBH-HPS (Shane, 1996). Vertical transmission to progeny can be best prevented by practicing effective biosecurity to control horizontal spread in commercial parent flocks and limit the introduction of additional pathogens (Dhillon, 1986).

**Vaccination:**

Vaccination programs involving the FAdV’s were largely initiated in response to the IBH outbreak in Australia and HPS in Pakistan and Latin American countries. The live vaccine manufactured from highly virulent strains of serotypes 8 was effective in controlling the outbreak situation in these countries. It was proven safe and effective broiler breeder progeny were vaccinated at eight to eleven weeks of age. An inactivated oil emulsion vaccine is available that protects against IBH and HPS in chickens. It is recommended for use in chicks older than eight weeks and is injected subcutaneously. It is recommended that grandparent flocks of breeders be vaccinated during grow out at eight to twelve weeks of age. Broilers should be vaccinated early in life.

**Targets for anti-adenovirus treatment:**

The number of virus-encoded proteins of adenovirus that have been used as potential targets for antiviral therapy is limited. As illustrated above, the majority of compounds having anti-adenovirus activity is inhibited by adenovirus DNA polymerase that is nucleoside or nucleotide analogues. Viral protease is a suitable target for development of antiviral drugs because adenovirus infectivity, maturity and uncoating are depends upon proper adenain activity (Pang et al., 2001). Numerous structural proteins of AdVs (IIIa, VI, VII, VIII, 55K) are produced as precursor’s which as such integrated in to
the virion. Precursors within the virions proteolytically cleaving protease at the final stage of morphogenesis (Weber, 1995). Blocking of proteolytic cleavage of viral protein is the possible target for chemotherapy of viral infection (Nosach et al., 2002). The activity of AdV proteinase was effectively inhibited by Nitric oxide, green tea catechins, and classic cysteine proteinase inhibitors such as iodoacetate (Cao et al., 2003).

**Cell culture and Antiviral drug discovery:**

In-vitro cell culture studies of chemopreventive agents is an important tools for testing the preliminary in vivo studies which provide data analysis cytopathic effects (CPE’s), plaque formation and virus yield on cell culture according to the analysis of AdV replication (Gordon et al., 1991). Although comparatively straight forward and economical, microscopic examination of CPE’s (as indicated by rounding and clumping of cells) can somewhat subjective. Many methods have been developed to measure the cell proliferation, including those based on direct counting of viable cells, measuring the metabolic activity and cellular DNA content. The test methods such as trypan blue dye exclusion using a hemacytometer traditional cell counting are simple and inexpensive but very time consuming and occasionally imprecise (Kanemura et al., 2002). Cytotoxicity quantify with 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) assay by infected cells viability (Kodama et al., 1996). However, the choice of method assessing the viability and proliferation may also significantly influence the quantitative evaluation of anti-proliferative activities of such as green tea.

**Vaccines:**

A vaccine is remarkably valuable in controlling viruses and the diseases they cause, but it has to follow certain prerequisites to be effective. First of all, a vaccine must be safe: its side effects must be minimal and it should induce protective immunity in the population as a whole, evoking innate, cell-mediated and humoral responses. Not every individual in a population needs to be immunised to stop viral spread, but a sufficient number must become immune
to prevent virus transmission. Protection provided by a vaccine should be long-term, meaning that more than one inoculation may be necessary in some cases. In practical terms, an effective vaccine should be biologically stable: there should be no genetic reversion to virulence and it should be able to survive storage and use in different conditions. Vaccines should also be easy to administer at low cost. Given the remarkable success of the smallpox and polio vaccines, it might seem reasonable to prepare vaccines against all viral diseases. Unfortunately, despite given the remarkable success of the smallpox and polio vaccines, it might seem reasonable to prepare vaccines against all viral diseases. Unfortunately, despite considerable progress in research, it is difficult to predict with confidence the efficacy or side effects of vaccines. Vaccines also require a new vaccine to be developed for each virus or virus strain, should be administered before or in some cases, shortly exposure to be effective, are not immediately available for emerging or developed threat virus, may have unforeseen undesirable effects, and are difficult to produce for some pathogens (e.g., HIV). (Boomker et al., 2005).

**Antiviral Drugs:**

Antiviral drugs are a class of medication used exclusively for treating viral infections. The emergence of antiviral drugs is the product of an extensive knowledge of the genetic and molecular function of viruses, major advances in the techniques for finding new drugs, and to face intense pressure to deal with HIV. The general idea behind antiviral drug design is to identify the viral proteins target, or parts of proteins, which can be disabled. These objectives should be common to many strains of a virus, or even between different virus species of the same family, if one has large drug effectiveness.

Once targets are recognized, drugs candidate can be selected. High throughput screening (HTS) allows a researcher to effectively conduct millions of biochemical, genetic or pharmacological tests in a short period of time. Through this process one can rapidly identify active compounds, antibodies or genes which modulate a particular biomolecular pathway. The results of these
experiments provide starting points for drug design and for understanding the interaction or role of a particular biochemical process in biology.

Traditionally, the search for new antiviral has been by HTS, but this is moving successfully towards a combination of HTS, to initially identify target compounds, and computer design software, to alter the conformation of the compounds to improve their action.

There are two main difficulties with antiviral drugs. First there is the problem that by the time clinical signs and symptoms appear in acute infections, virus replication has reached such a peak that the antiviral has little therapeutic effect. The other problem is that virus multiplication is tied so intimately to certain cellular processes that most antivirals cannot discriminate between them. However, viruses do have unique features, so specific antiviral should be able to serve as effective chemotherapeutic agents. A further problem is the selection of drug-resistant mutants any naturally-arising mutant that happens to have a selective advantage will rapidly outgrow wild-type virus and become dominant. The answer to this problem is to use two or more drugs simultaneously.

An antiviral would be effective if it inhibited any stage of virus multiplication: attachment, replication, transcription, assembly or release of progeny virus particles. Most of the antiviral now available are designed to help deal with HIV; Herpesvirus, which is best known for causing cold sores but actually covers a wide range of diseases; and HBV and HCV, which can cause liver cancer.

**CONCLUSIONS:**

Literature reported to date it is concluded that tea is a dietary source of biologically active compounds that have different biological and pharmacological activities including antioxidant anti-inflammatory, anticancer, antibacterial and antiviral activity. Most of these activities contributed to polyphenolic compounds known as catechins. In summary, the antimicrobial activity of tea catechins is mainly exerted by binding directly to the structure of
the peptide components of bacteria, viruses and enzymes. Antiviral activities of
green tea, which directly inactivate the virus, interact with DNA polymerase
and reverse transcriptase of HIV. It also prevent the production of
proinflammatory, prostaglandin, TNF which contribute to disease symptom
during viral infections.
CHAPTER: 3

MATERIALS AND METHODS

Viral isolate:
The FAdV-4 isolate used in this study was acquired from Institute of Microbiology, University of Agriculture Faisalabad, Pakistan. The virus isolate was obtained from outbreak of IBH-HPS liver sample. The hexon gene of the virus amplified by polymerase reaction (PCR). The 730 kb PCR product was analyzed by nucleotide sequencing, which was submitted to GenBank (Accession # DQ 264726). Liver from the birds in the IBH-HPS outbreak contained FAdV-4, which was confirmed by sequence BLAST analysis of gene and a phylogenetic tree constructed and published (Mansoor et al., 2009). The same isolated and confirmed FAdV-4 was used for the present study.

Vero cell line:
The African green monkey kidney cells (Vero) was obtained from Centre for Applied Microbiology and Research (CAMR) are shipped on dry ice in cryopreservation vial (ECACC 84113001, ECACC, Salisbury, Wiltshire: Catalogue no. ATCC CCL81). Upon receiving of frozen cells, the cells were stored in liquid nitrogen vapor at -196°C.

Materials and Reagents for cell culture:
- Dulbecco’s modification of Eagle medium (DMEM) supplemented with heat inactivated fetal bovine serum (FBS), 5%, 10% and 20% was added in maintenance, growth media and preservation media and store at 4°C.
- FBS was aliquoted and stored at -20°C.
- Trypan blue Stain (0.4% w/v trypan blue in PBS) filter sterilized when use for the determination of the cell viability.
- Penicillin-streptomycin solution 100X stock solution and amphoteracin B at 0.2 mg/ml was used to avoid the contamination.
- Dimethyl sulfoxide (DMSO) for the preservation of the cells which is tissue culture grade.
- Sterile 25 cm² tissue culture flask with vented caps
- 70% solution of ethanol required for laminar flow hood decontamination and objects carried in to hood.
- Filter sterilizes Dulbecco’s Phosphate buffer saline solution (DPBS) without Ca++ and Mg++.
- Sterilized 1X trypsin-EDTA solution prepared in DPBS without Ca++ and Mg++.
- Appropriate Cryovial for freezing at liquid nitrogen.

**Thawing of Cryopreserved Vero cells:**
- Growth medium was prepared in biological safety cabinet containing 10% heat inactivated FBS and growth medium was warmed at room temperature.
- Frozen vial of cells were removed from the liquid nitrogen storage container and thaw the vial of Vero cells by gently swirling in water bath at 37°C. The cryovial O ring and cap was out of the water bath due to avoid contamination.
- In Biological safety cabinet, a sterile glass pipet was use to transfer the vial content in to growth medium containing in 15 ml conical tube.
- The frozen cryovial was containing DMSO, after thawing the cells remove DMSO and the cells was diluted to avoid harmful effects of DMSO.
- The pellet of the cells was obtained through centrifugation at 1200 rpm (300g) for 5 minutes.
- The supernatant was discarded and the cells were resuspended in 25 cm² tissue culture flask containing 7 ml of growth medium.
- The suspension of Vero cells was transfer vented cap tissue culture flask.
- The flask was incubated carbon dioxide (CO₂) incubator (Sanyo, Japan) at 5% CO₂ and 37°C temperature.
- The cultures were examined daily under inverted microscope (Olympus CK 40, Japan) to ensure that the culture was not contaminated and cells were growing.
Subculturing of Vero cells:

Vero cell culture was double at about 24 hours. Vero cell was usually needed to passage 2-3 times per week (Freshny, 2000).

- Growth medium was removed from confluent monolayer of Vero cells.
- Monolayer of cells was washed with DPBS without Ca++ and Mg++ twice to completely remove the growth medium.
- 5 ml of trypsin-EDTA solution was added to the flask and the flask was incubated for 3 minutes and the flask was gently shake and tap until the cells were detached from the flask.
- Action of trypsin-EDTA was stopped by adding 5 ml of media containing 10% of FBS
- Cells were wash down in the media pipetting the cells to make single suspension
- Cell suspension was removed from the flask and transfer to 15 ml conical sterilized flask.
- At room temperature, the cells were centrifuged at 200× for 5 minutes.
- The cells were resuspended in 5 ml DMEM with 10% FBS after discarding the supernatant.
- A 100 µl was removed to check the viability and cells were counted with haemocytometer.
- 3×10^4 cells/cm² of cells was seeded into a new flask containing pre-warmed growth medium at split ratio of 1-3 for subculturing of Vero cell.
- The flasks were incubated at 5% CO₂ in CO₂ incubator (Sanyo, Japan) at 37°C and monitor the cell for growth every day under inverted microscope, repeat the protocol when cells was reached the >90% confluency.

Determination of cell number and cell viability:

The densities and cell viability was checked (Freshny, 2000).

- The culture dish was swirl gently to distribute the cell suspension evenly.
From evenly distributed cell suspension, an aliquot (100 µl) was removed under sterilized conditions.

The 100 µl of trypan blue a viable stain and 100 µl cells were prepared that was given dilution factor of 2.

The surface of haemocytometer and cover slip was cleaned with 70\% isopropanol and allowed to dry.

The chamber of the haemocytometer was filled with dilution of well mixed cell and was observed under light microscope using 20 x lens.

The number of cells were counted viable (bright clear) and nonviable (blue) in 1-mm².

The no of nonviable and viable cells and viable cells percentage were calculated by the following formulas where (A) is the mean number of viable cells counted, (B) is the mean number of nonviable cells counted, C is the dilution factor (it was 2), (D) is the correction factor supplied by the hemocytometer manufacturer (this is the number required to convert 0.1 mm³ into milliliters; it is usually 10⁴).

\[
\text{Concentration of viable cells (per ml) = 102} \times 20 \times 2 \\
\text{Concentration of nonviable cells (per ml) = 20} \times 2 \times 10^4 \\
\text{Total number of viable cells = 4080} \times 1 = 4080 \\
\text{Total number of cells = 4080+ 3600} \\
\text{Percentage viability= (number of viable cells} \times 100)/\text{total cell number (88.23\%)}
\]

**Cryopreservation of Vero cells:**

To avoid the loss of cell line, the cell was cryoperversed for future use. The continuous growth was increases the chance of contamination by bacteria.

- The cultures were observed under inverted microscope to assess the cell density and absence of bacterial and fungal contamination was confirmed.

- The density and viability of cell was determined by removing the small aliquot of cells. For cryopreservation the cells were in log growth stage with more than 90\% viability.
The cryovial was prepared by indicating the name of cell line, the number of cells per vial, the date and the passage number on the surface of the vial with permanent marker.

Required freezing medium was prepared which contain 1 ml of DMSO and 9 ml of DMEM in 15 ml conical flask.

Growth medium was removed from the flask of confluent monolayer of Vero cells.

Cells were washed with 5 ml of DPBS twice to remove the media from the flask.

For detachment of cell, 5 ml trypsin-EDTA solution was added and cells were incubated for 3 minutes to detach the cells from the flask.

To inactivate the trypsin-EDTA action 5 ml of media with 20% FBS was added in the flask.

The cells were washed with the media and the cells were gently pipetting to make single cell suspension.

Suspension of the cells was transferred to sterilized 15 ml conical flask and centrifuged the cells for 5 minutes at 200 × g.

Supernatant was removed and cells were resuspended in 10 ml of DMEM with 20% FBS and 10% DMSO.

1 ml of resuspended cells was added into each cryovial.

Cells were freeze slowly in -80°C and were stored at -196°C in liquid nitrogen.

**Propagation and confirmation of virus isolate:**

**Virus stocks:**

The fowl adenovirus-4 virus stock was prepared by inoculating monolayer of Vero cells in 25-cm² tissue culture flask with virus 1:5 to 1:10 in 1 ml DMEM containing 2% FBS. The flask was placed in an incubator at 37°C to allow virus adsorption. After 1 hour 4 ml of DMEM supplemented with 2% FBS was added and the cells were allowed to continue propagating at 37°C for 7 days until the CPE’s are confirmed. The cells and supernatant were then harvested by gentle pipetting. Cell debris was removed by centrifugation at...
1500 x for 5 minutes. The viral supernatant was collected in aliquots of 1 ml each and stored at -80°C till further use. These viral supernatant was subjected to reverse passive haemagglutination (RPHA) and agar gel precipitation test (AGPT) for confirmation, 

**Hyperimmune serum:**

Serum against FAdV-4 was raised following Hussain *et al.*, 2004. Briefly, ten one-day-old chicks (Sabir Poultry Breeders, Lahore) were reared in the animal house, Institute Microbiology, UAF. The birds at 10 days of age were negative for maternal antibodies then these were primed with killed HPSV vaccine (Sana Lab) and were given first booster on day 20 with a double dose of oil based killed HPSV vaccine and second booster on day 30 with triple dose of the same vaccine. On 14th day of the last booster the blood samples were collected and the sera was separated and stored at –20°C till used.

**Sensitization of Sheep Erythrocytes:**

Using 0.5ml of sodium citrate solution 5 ml of sheep blood was collected and centrifuged to sediment the cells, and the supernatant was discarded. This procedure was repeated thrice and finally 2% sRBCs suspension in normal saline was prepared. An equal volume of 1:20,000 freshly prepared tannic acid solution in 2% washed sRBCs was added. The mixture was incubated at 37°C for 10 minutes then washed with normal saline and finally 2% sRBCs suspension was made to sensitize with hyper-immune serum against FAdV-4. Tanned sRBCs were mixed with equal volume of hyper-immune serum against FAdV-4 and allowed coating at room temperature for 10 minutes (Nachimuthu *et al.*, 1995). Excess of the antibodies were washed off by centrifugation of the mixture at 2000 rpm for three minutes.

**RPHA test:**

The RPHA test was performed following the method of Nachimuthu *et al.*, (1995) with modifications. The test was carried out in 96 well (U-shaped bottom) microtitration plates using two fold dilutions of the antigen in 50 µl of Phosphate buffer saline (PBS) followed by addition of 50 µl of 1% sensitized sRBCs. Then the microtitration plates were incubated at 37°C for 30 minutes
and were examined. A positive reaction consisted of marked haemagglutination, while a negative reaction was evidenced by button formation at the bottom of the well.

**AGPT Test:**

The test was performed following the procedure described by Cullen and Wyeth (1975) with some modifications. The test was standardized for 0.9% Noble Agar composition in distilled water, temperature 37°C, pH of the gel (7.0) and time for incubation (24-48 hours).

**Virus titration by plaque assay:**

Fowl adenovirus serotype 4 was titrated by plaque assay using Vero cells. Briefly, Vero cells were seeded in 6-well plates (6×10^5 cell/ml) in DMEM with 10% FBS for 24 hours at 37°C, the cells were confluent and there was no hole in the monolayer. After cell propagation growth medium was removed. The virus preparation was diluted in a series of ten-fold dilutions in DMEM supplemented with 2% FBS, and cells were inoculated with each virus stock dilution (1ml/well). Duplicate wells of cells were set up for each dilution. The inoculated cells were further incubated to allow adsorption for 2 hours at 37°C, 5% CO₂ were grown in 6-well plates until 90% confluence. The cells with the virus inoculum were incubated for 2 h (37°C, 5% CO₂) on a rocking platform to allow adsorption of virus onto the cells. The virus inoculum was then removed and 3 ml of overlay, which consists of 0.5% of low-melting agarose and 2% FBS in DMEM, were added to each well.

The cells were incubated (at 37°C, 5% CO₂) for 8 days without any mechanical agitation. When plaques were distinctly formed, the medium was aspirated off and the monolayers were fixed by adding 2 ml of 5% formaldehyde for overnight to penetrate the agarose, after which the fixative solution was washed away with water. Areas of cell lysis and therefore sites of virus infection or plaques were visualised by crystal violet staining, by incubating the fixed cells with a solution of 1% crystal violet for 20 min, on a rocking platform at room temperature. After briefly washing the monolayers with water, plaques were observed as "holes" or unstained areas of the monolayer. Plaques were
counted and the titre was estimated (plaque-forming-units per ml of virus preparation; pfu/ml), taking into account the dilutions made. Viral titer was calculated by multiplying the mean number of plaques per well by the serial dilution value to determine the concentration in 1 ml of the virus preparation was assayed.

**Preparation of green tea extract:**

Green tea extract was prepared by addition of 20 ml boiling double distilled deionized water (d$_3$H$_2$O) to 1 gm of dry tea leaves for ten minutes at 75°C in a sterilized and sealed glass container, was centrifuged and filtered through a 0.22µ filter. Green tea extract was lyophilized for further use.

The green tea catechins *i.e.*, EGCG (Cat#4143), ECG (Cat #3893) and EGC (Cat #3768) were purchased from Sigma-Aldrich.

**Plaque reduction assay:**

Vero cells confluent monolayer was cultured in tissue culture plates having 24-wells with a cells density of 1× 10$^5$ cells/cm$^2$. A 500 plaque forming units per ml (PFU/ml) of FAdV-4 was to infected the confluent monolayer of the Vero cells per well (Das *et al.*, 1999). After allowing adsorption of virus at 37°C for 2 h, the viral inoculums was decanted and the cells were washed with DPBS. Overlay agar medium supplemented with 0.5% low melting agarose and 2% FBS in DMEM with various concentration of test compounds 0, 50, 100, 200 µg/ml GTE and at 0, 30, 60, 120 µM/ml of ECGC, EGC and ECG respectively were added.

Incubation of the cultures were kept for 3 days at 37°C in the presence of 5% CO$_2$ and the monolayer was fixed by using 5% formaldehyde solution for 30 min. The agarose was removed by rinsing water and was stained by using 1% (w/v) crystal violet for plaque visualization. The percentage of plaque inhibition was calculated relative to the control. The antiviral activity of the test compounds was determined by the following formula:

\[
\text{Percentage inhibition} = \left[ 1 - \frac{\text{number of plaque}_{\text{tested}}}{\text{number of plaque}_{\text{control}}} \right] \times 100\% 
\]
The minimal concentration of different compounds to reduce the 50% plaque number (EC$_{50}$) was calculated by regression analysis of the dose response curves generated from these data.

**Virus yield reduction assay:**

The production of infectious virus particles (virus yield) on a time course basis after infection with FAdV-4 was carried out. Vero cells were grown as monolayers in tissue culture plates which contain 12-wells. The confluent monolayer of Vero cells were formed, the medium was decanted and washed the monolayers with DPBS. The cells were infected with 10$^6$ PFU/ml of FAdV-4 per well. The dishes were placed on shaker for 45 minutes at room temperature for regular overturning in drug-free conditions for proper adsorption of the virus. The viral inoculum was removed and monolayers were washed three times with DPBS. The growth medium containing 0, 50, 100, 200 µg/ml of GTE and at 0, 30, 60, 120 µM/ml of ECGC, EGC and ECG, respectively was added in 12 well plates. The plate was incubated in 5% CO$_2$ at 37°C in humidified atmosphere. At periodic intervals (8, 24, 48 h) after infection, duplicate samples from each group were collected separately by scraping the ells off the plate and freezing the cells and supernatant at -70°C. Freeze thaw sample was centrifuged at 1500 rpm to remove the cell debris. The supernatant was serially diluted and titrated in Vero cell monolayer to ascertain the virus titer. Throughout the experiment, as a control infected cells were incubated in catechins free medium.

**Cytotoxicity assay:**

The cell viability assay is based on the reduction of MTT 3-(4, 5-dimethylthiazol-2-yl) -3, 5-diphenyl tetrazolium bromide), by the viable cells mitochondrial dehydrogenase enzyme, to form dark blue formazan particles and read spectrophotometrically. The clorimetric MTT assay was executed in 96-well plates. Vero cells at a concentration of 5×10$^4$ cells were seeded in 96-well plate with 100 µl suspension of cells in each well. When the cells anchored to the plate, the media was removed from the plate. A fresh media without serum was added containing various concentration test compounds 0, 50, 100,
150, 200, 250 µg/ml GTE and at 0, 30, 60, 120, 180, 240 µM/ml ECGC, EGC, and ECG respectively. Duplicate wells were set for each sample. The cells with test compounds were incubated for 48 hours. Subsequently, the cells were processed within MTT (Liu et al, 2003). Following incubation at 37°C for 48 hours, 20 µl of 5 g/l of MTT was added in each well and the cells were incubated for another 4 hours. The MTT was discarded and the formazan particles were solubilized by addition of warm 200 µl of DMSO in each well. The absorbance of the culture at wavelength of 490 nm was measured. Control cells were cultured in the same way in conditions without drugs. Data were calculated as percentage of inhibition by the following formula.

\[
\text{Inhibition}\% = \left[ 100 - \left( \frac{\text{OD}_{t}}{\text{OD}_{s}} \right) \times 100 \right] \%
\]

\(\text{OD}_{t}\) and \(\text{OD}_{s}\) indicates the optical density of the test substances and the solvent control, respectively. The concentration of 50% cellular Cytotoxicity (CC50) of test substances was calculated.

**Experimental Animals:**

Three hundred and twenty day-old 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.

**Experimental Design:**

The chicks were divided in to four groups at 14 day of age, having 80 birds in each group. All the groups were offered feed and water *ad libitum*. The birds of all the groups were treated as follows.
Table 1: Different groups Treated with different products at different concentration along with control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (For six days)</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>EGCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1 30 mg/l</td>
<td>DW</td>
</tr>
<tr>
<td></td>
<td>A2 60 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A3 120 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A4 Control</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>ECG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B1 30 mg/l</td>
<td>DW</td>
</tr>
<tr>
<td></td>
<td>B2 60 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B3 120 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B4 Control</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>EGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C1 30 mg/l</td>
<td>DW</td>
</tr>
<tr>
<td></td>
<td>C2 60 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C3 120 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C4 Control</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>GTE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D1 50 mg/l</td>
<td>DW</td>
</tr>
<tr>
<td></td>
<td>D2 100 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D3 150 mg/l</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D4 Control</td>
<td></td>
</tr>
</tbody>
</table>

Protection against FAdV-4:

To measure the efficacy of different concentration of catechin and green tea extract, the birds from all the groups were challenged orally with $10^{4.8}$ mean egg infective dose (EID$_{50}$) per chicken with the FAdV-4, given at 14 days of age. At day of challenge, treatment was given for 6 days through drinking water. Clinical signs and mortality was examined daily for 7 days.
**Body weight and Survival rate of broiler chicks:**

The birds were weighed daily throughout the treatment period after challenge in each group. Survival rate of broilers were noted in each groups throughout the treatment with different test compounds,

**Gross and histopathological lesion scoring:**

The dead and survived birds on post mortem at the end of treatment were subjected to histopathological lesion scoring. The histopathological lesions observed in liver and kidney were subjectively evaluated and scored from 0 to 3 were done on the basis of following cateria. No lesion = 0, mild lesions = 1, moderate lesions = 2, severe lesions = 3. The bursal score was done on the basis of following citeria.

0: No lesion

1: Scattered lymphoid necrosis

2: Sever lymphoid depletion in some follicles

3: Lymphoid depletion in all most follicles

4: loss of lymphoid depletion and increase in inter follicular connective tissues

**Procedure for histopathology:**

The histopathological by following the method of Bancroft and gabble, (2008) as described below.

- Neutral buffered formalin solution (Formalin 37% (w/v) 100 ml, distilled water 900 ml, Sodium di-hydrogen Phosphate 4.0 g, disodium Hydrogen Phosphate 6.5 g) were used for the fixation of tissue. The tissue was completely dipped in solution for about 10-15 days at room temperature to prevent postmortem changes and facilitate the proper staining and hardening of tissue texture.

- After fixation of tissue 5 mm slices were taken from selected organ and washed in running water overnight to remove fixative. After that slices of tissues were placed in different solution with different concentration for purpose of dehydration, clearing and infiltration.
- Paraffin wax was used for tissues embedding purposes, it form homogenous mass after infiltration. Liquid paraffin was poured around the tissue and allows it to solidify.
- After embedding, the tissue was sectioned to 3-4 µm thickness using a rotary microtome and the sections were mounted on the slides.
- The sectioned tissues were mounted on the glass slides with the help of adhesive mixture which was Mayer’s egg albumin.
- The sections were floated on a hot water at 53°C in water bath. Dried the slide mounted sections at 37°C for 24 hrs.
- After drying, they were incubated at 45°C for 30 minutes
- Tissues were stained with hematoxylin and eosin

**Organ to Body Weight Ratio (Liver, spleen and kidney):**

The liver, spleen and kidney from survived and dead birds from each group were collected and each organ was weighed to calculate the organ to body weight ration during the treatment period. Actual weights (g) of the organs (Liver, spleen and kidney) were recorded within half an hour of slaughtering. Since organ weight is directly related to body weight, percent weight of these organs (liver spleen and kidney) to the body weight was also calculated by (organ weight/body weight) × 1000 (Khan *et al.*, 2012).

**Statistical Analysis:**

Student’s unpaired *t*-test was used 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.
CHAPTER-4

RESULTS

IBH-HPS is a recently emerged immunosuppressive disease of 3-6 week old broilers, characterized by sudden onset, high mortality, and typical hydropericardium and swollen and friable livers with large basophilic intranuclear inclusion bodies in hepatocytes (Balamurugan and Kataria, 2004).

In order to control IBH-HPS there is an extensive use of liver homogenates in which the virus is inactivated by certain chemical substances like formalin (Anjum, 1990; Hussain et al., 1999; Roy et al., 1999). These so called vaccines are only the parameter for immunizing the birds from IBH-HPS and immune response provoked by these vaccines is not always consistent or predictable (Khan et al., 2005).

The present study was aimed to evaluate the antiviral effect of GTE and its isolated polyphenolic compound against FAdV-4 which is the causative agent of IBH-HPS in broilers in-vitro against FAdV-4 and in-vivo. Antiviral activity of these compounds was evaluated and compared their effects in-vitro in cell culture and in-vivo in poultry.

Propagation of FAdV-4 on Vero cells and there was adaptation of virus on the Vero cells (plate 1) and after the appearance of CPE’s. Than this flask was subjected to plaque assay to determine the plaque forming unit (pfu/ml) of the virus (Plate 2)
**Figure 2:** Normal growth of Vero cells 90% confluent monolayer

**Figure 3:** Appearance of CPEs after 6 days infection of FAdV- 4
Cytotoxicity assay:

Cytotoxicity assay was carried out in 96 wells plate of Vero cells. Vero cells were grown in 96 wells plate and treated with different compounds at different concentrations. The plate was incubated and MTT reagent was added after incubation. Than the dimethyl sulfoxide (DMSO) was added in the treated plate and the reading was taken in ELISA reader at 490 nm. The GTE showed minimum cytotoxic effect towards the Vero cells.

![Assessment of viable cells with MTT assay](image)

**Table 2:** Percentage inhibition cell by EGCG

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8862±0.01097a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.4953±0.00802b</td>
<td>44.0975±1.17687</td>
<td>49.815</td>
</tr>
<tr>
<td>60</td>
<td>0.4153±0.1210c</td>
<td>53.1155±1.94999</td>
<td>49.939</td>
</tr>
<tr>
<td>120</td>
<td>0.3973±0.01201d</td>
<td>55.1681±0.89934</td>
<td>52.044</td>
</tr>
<tr>
<td>180</td>
<td>0.2290±0.02364e</td>
<td>74.1338±3.00582</td>
<td>43.671</td>
</tr>
<tr>
<td>240</td>
<td>0.1983±0.00902f</td>
<td>77.6096±1.26324</td>
<td>83.893</td>
</tr>
</tbody>
</table>

Control = without test compounds, 30 = 30µM of EGCG, 60 = 60µM of EGCG, 120 = 120µM of EGCG, 180 = 180µM of EGCG and 240 = 240µM of EGCG
### Table 3: Percentage inhibition cell by EGC

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8862±0.01097a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.4975±0.00477b</td>
<td>43.8499±1.18381</td>
<td>56.279</td>
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<tr>
<td>60</td>
<td>0.4670±0.1647c</td>
<td>47.2984±1.84354</td>
<td>36.696</td>
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<tr>
<td>120</td>
<td>0.4093±0.01710d</td>
<td>53.8106±1.75380</td>
<td>40.656</td>
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<tr>
<td>180</td>
<td>0.3086±0.17999e</td>
<td>65.1674±2.09009</td>
<td>47.476</td>
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<tr>
<td>240</td>
<td>0.1943±0.0061f</td>
<td>78.0692±0.68714</td>
<td>95.432</td>
</tr>
</tbody>
</table>

Control = without test compounds, 30 = 30µM of EGC, 60 = 60µM of EGC, 120 = 120µM of EGC, 180 = 180µM of EGC and 240 = 240µM of EGC

### Table 4: Percentage inhibition cell by ECG

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8862±0.01097a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0.4420±0.03816b</td>
<td>50.1366±3.98562</td>
<td>19.377</td>
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<tr>
<td>60</td>
<td>0.4078±0.02167c</td>
<td>53.9759±2.51984</td>
<td>34.112</td>
</tr>
<tr>
<td>120</td>
<td>0.3650±0.3764d</td>
<td>58.7941±4.44310</td>
<td>23.023</td>
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<tr>
<td>180</td>
<td>0.3067±0.00451e</td>
<td>65.3942±0.22582</td>
<td>84.629</td>
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<tr>
<td>240</td>
<td>0.2037±0.08609f</td>
<td>77.0185±9.63740</td>
<td>13.620</td>
</tr>
</tbody>
</table>

Control = without test compounds, 30 = 30µM of ECG, 60 = 60µM of ECG, 120 = 120µM of ECG, 180 = 180µM of ECG and 240 = 240µM of ECG
Table 5: Percentage inhibition cell by GTE

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8862±0.01097a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.4933±0.00709c</td>
<td>44.3249±0.96541</td>
<td>52.083</td>
</tr>
<tr>
<td>100</td>
<td>0.4943±0.00681b</td>
<td>44.2128±0.86455</td>
<td>52.570</td>
</tr>
<tr>
<td>150</td>
<td>0.4163±0.1012e</td>
<td>53.0181±1.01023</td>
<td>54.535</td>
</tr>
<tr>
<td>200</td>
<td>0.4320±0.5048d</td>
<td>51.2652±5.4457</td>
<td>15.228</td>
</tr>
<tr>
<td>250</td>
<td>0.3093±0.01206f</td>
<td>65.0906±1.37848</td>
<td>61.297</td>
</tr>
</tbody>
</table>

Control = without test compounds, 50 = 50µg of GTE, 100 = 100µg of GTE, 150 = 150µg of GTE, 200 = 200µg of GTE and 250 = 250µM of GTE
Figure 5: Over all cell inhibition % age after treatment of normal cell with EGCG, ECG and EGC at 30, 60, 120, 180 and 240 µM/ ml

![GTE Graph](image)

Figure 6: Over all cell inhibition % age after treatment of normal cell with GTE at 50, 100, 150, 200 and 250 µg/ ml

**In-vitro antiviral activity was evaluated through Plaque inhibition assay:**

The concentration of the above mentioned compounds were selected which were tolerated well to normal cells in MTT assay. The percentage inhibition was 23.0405±5.02165 at 30 µM/ml, 24.0741±2.31296 at 60 µM/ ml and 36.8421±2.10526 at 120 µM/ ml by Epigallocatechin gallate as presented in Table 5, figure 3 and plate 4. The percentage inhibition of plaques by EGC showed 13.3376±2.07497 at 30 µM/ ml, 12.5926±2.79623 at 60 µM/ml and 28.7719±3.21584 at 120 µM/ml concentrations as shown in Table 6, figure 3 and plate 5. The ECG compound inhibited plaques at 30 µM/ ml was 21.1988±6.81299, 17.0370±2.79623 at 60 µM/ ml and 27.7193±3.21584 at 120 µM/ml as described in Table 7, figure 3 and plate 6. The green tea extract inhibited 44.7576±0.44197 at 50 µg/ml, 77.7583±2.90658 at 100 µg/ml and 62.4561±4.25416 at 150 µg/ ml as shown in Table 8, figure 3 and plate 7.
### Table 6: Plaque inhibition percentage by EGCG

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Plaque count</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.333±2.51661a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>71.000±3.60555b</td>
<td>23.040±5.02165</td>
<td>8.404</td>
</tr>
<tr>
<td>60</td>
<td>68.333±2.08167c</td>
<td>24.074±2.31296</td>
<td>12.728</td>
</tr>
<tr>
<td>120</td>
<td>60.000±2.00000d</td>
<td>36.842±2.10526</td>
<td>17.422</td>
</tr>
</tbody>
</table>

Control = without test compounds, 30 = 30µM of EGCG, 60 =60µM of EGCG, 120 = 120µM of EGCG

### Table 7: Plaque inhibition percentage by EGC

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Plaque count</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.333±2.51661a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>80.000±2.00000b</td>
<td>13.337±2.07497</td>
<td>6.645</td>
</tr>
<tr>
<td>60</td>
<td>78.667±2.51661c</td>
<td>12.593±2.79623</td>
<td>6.651</td>
</tr>
<tr>
<td>120</td>
<td>67.667±3.05505d</td>
<td>28.772±3.21584</td>
<td>10.794</td>
</tr>
</tbody>
</table>

Control = without test compounds, 30 = 30µM of EGC, 60 = 60µM of EGC 120 = 120µM of EGC

### Table 8: Plaque inhibition percentage by ECG

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Plaque count</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.333±2.51661a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>74.667±2.51661b</td>
<td>17.037±2.79623</td>
<td>8.598</td>
</tr>
<tr>
<td>120</td>
<td>68.667±3.05505d</td>
<td>27.719±3.21584</td>
<td>10.356</td>
</tr>
</tbody>
</table>

Control = without test compounds, 30 = 30µM of ECG, 60 = 60µM of ECG 120 = 120µM of ECG
Table 9: Plaque inhibition percentage by GTE

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Plaque count</th>
<th>%age inhibition</th>
<th>t vs control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.333±2.51661a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>51.000±1.00000b</td>
<td>44.757±0.44197</td>
<td>26.437</td>
</tr>
<tr>
<td>100</td>
<td>20.333±2.08167d</td>
<td>77.758±2.90658</td>
<td>38.184</td>
</tr>
<tr>
<td>150</td>
<td>35.667±4.04145c</td>
<td>62.456±4.25416</td>
<td>20.616</td>
</tr>
</tbody>
</table>

Control = without test compounds, 50 = 50µg of GTE, 100 =100µG of GTE, 150 = 120µM of EGC

Figure 7: Plaque inhibition assay by EGCG
**Figure 8:** Plaque inhibition assay by EGC

**Figure 9:** Plaque inhibition assay by ECG
**Figure 10:** Plaque inhibition assay by GTE

**Figure 11:** Percentage inhibition of plaques by all tested compounds at 30 µM/ml, 60 µM/ml, and 120 µM/ml, of EGCG, EGC, ECG and 50 µg/ml, 100 µg/ml and 150 µg/ml concentrations of GTE
**Virus reduction assay:**

Different concentration of EGCG, ECG, EGC and GTE were used in the current study and at 8, 24 and 36 hours interval the reduction in the virus yield was observed. Maximum virus yield reduction was found in GTE at 36 hours. The plaque forming units (PFU/ml) were calculated and subjected to statistical analysis. EGCG reduced the plaques with increasing time and 120 µM/ml concentration reduced virus yield considerably as shown in figure 4. EGC reduced the virus yield at 120 µM/ml concentration as presented in figure 5. ECG also had reduced the virus yield maximum at higher concentration as shown in figure 6. The green tea extract reduced the virus yield maximum at 100µg/ml concentration as compared to 50µg/ml and 150µg/ml concentration as shown in figure 12.

![Figure 12: Inhibitory effect of EGCG on virus yield at 8, 24 and 36 (hrs) post-infection](image-url)
Figure 13: Inhibitory effect of EGC on virus yield at 8, 24 and 36 (hrs) post-infection

Figure 14: Inhibitory effect of ECG on virus yield at 8, 24 and 36 (hrs) post-infection
**Figure 15:** Inhibitory effect of GTE on virus yield at 8, 24 and 36 (hrs) post-infection

**Calculation of EC₅₀, CC₅₀ and SI:**
After in-vitro study of these compounds EC₅₀, CC₅₀ was calculated and then SI was calculated. The green tea extract had highest SI as shown in Table 9. The other compounds had very low SI. The EC₅₀ of EGCG, EGC, ECG and GTE was 207.12, 241.70, 392.52 and 34.80 respectively. The CC₅₀ of EGCG, EGC, ECG and GTE was 59.56, 70.88, 34.95 and 100.23 respectively. SI was maximum in case of GTE (3.165) followed by EGC (0.32), followed by EGCG (0.28) and lowest SI was found in ECG (0.089) as presented in Table 9.
Table 10: Anti-adenovirus activities and cytotoxicity of the EGCG, ECG, EGC and GTE

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>SI&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>207.12</td>
<td>59.56</td>
<td>0.28</td>
</tr>
<tr>
<td>EGC</td>
<td>241.70</td>
<td>70.88</td>
<td>0.32</td>
</tr>
<tr>
<td>ECG</td>
<td>392.52</td>
<td>34.95</td>
<td>0.089</td>
</tr>
<tr>
<td>GTE</td>
<td>34.80</td>
<td>100.23</td>
<td>3.165</td>
</tr>
</tbody>
</table>

a: EC<sub>50</sub> represents the concentration of test compound necessary for a reduction in plaque number by 50% relative to control without test compound

b: CC<sub>50</sub> represents cellular toxicity to uninfected Vero cells as determined by MTT assay

c: SI is the ratio of CC<sub>50</sub> values to EC<sub>50</sub> values

**Survival rate:**

In-vitro evaluated compounds were trialed in live birds and the birds were treated with different concentrations of all the compounds tested after challenge of FAdV-4 at day 14 of age. Survival rate was calculated by observing the treated birds daily up to 7 days. Highest survival rate was observed in the birds treated with GTE followed by EGCG followed by EGC followed by ECG as shown in table 10 and figure 8. Among different concentration of green tea extract, a 100 mg/ml concentration gave maximum survival rate followed by 150 mg/ml and 50 mg/ml concentrations.
Table 11: *In-vivo* survival rate of broiler chickens

<table>
<thead>
<tr>
<th>Concentration</th>
<th>ECG</th>
<th>EGC</th>
<th>EGCG</th>
<th>GTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>30, 50</td>
<td>55</td>
<td>59</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>60, 100</td>
<td>65</td>
<td>65</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>120, 150</td>
<td>60</td>
<td>70</td>
<td>75</td>
<td>85</td>
</tr>
</tbody>
</table>

Figure 16: Survival rate of Broiler chickens after treatment by giving challenge with FAdV-4

Organ to body weight ratio:

Another parameter was studied in this study in in-vivo trials of broiler chickens. The birds were selected randomly from each group of birds treated with different compounds and subjected to organ to bodyweight ratio and lesion scoring. The birds were slaughtered after weighing and then liver bursa and spleen weights were measured, ratio was calculated and compared with control group. The birds treated with ECG have significantly higher lesion score at all concentrations used. The liver, bursa and spleen weight ratios were also significantly higher at all concentrations in comparison with control as shown in table 11. In EGC treated group significantly higher lesion score was found at
all concentrations used. The liver, bursa and spleen weight ratios were also significantly higher at all concentrations as compared to control group as shown in table 12. The EGCG treated group had lower lesion score as compared to ECG and EGC treated groups but significantly different from GTE treated group. The organ to body weight ratios statistically significant at all concentrations as shown in table 13. The green tea extract treated group had lowest lesion scoring as compared to all other groups but different concentration had significant effect on lesion score. A concentration of 100 mg/ml was found non-significant statistically in comparison with control. No any significant difference was found statistically among liver bursa and spleen weight ratios as presented in table 14.

Table 12: Organ to body weight ratio (Liver, Bursa, Spleen) and gross lesion score (Liver) after treatment of ECG

<table>
<thead>
<tr>
<th>Organ to body weight ratio</th>
<th>Liver</th>
<th>Bursa</th>
<th>Spleen</th>
<th>Lesion Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.100±0.1581d</td>
<td>0.3500±0.0158a</td>
<td>0.1780±0.01304a</td>
<td>0.00±0.00c</td>
</tr>
<tr>
<td>30</td>
<td>7.800±0.1871a</td>
<td>0.2160±0.01816c</td>
<td>0.1020±0.0083c</td>
<td>3.80±0.45a</td>
</tr>
<tr>
<td>60</td>
<td>7.380±0.1304b</td>
<td>0.2860±0.0040b</td>
<td>0.1320±0.0084b</td>
<td>3.60±0.55a</td>
</tr>
<tr>
<td>120</td>
<td>6.900±0.1000c</td>
<td>0.2960±0.0114b</td>
<td>0.1200±0.0071b</td>
<td>2.80±0.45b</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>7.045±0.6621</td>
<td>0.2870±0.5069</td>
<td>0.1330±0.0301</td>
<td>2.55±1.61</td>
</tr>
<tr>
<td>P value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 13: Organ to body weight ratio (Liver, Bursa, Spleen) and gross lesion score (Liver) after treatment of EGC

<table>
<thead>
<tr>
<th>Organ to body weight ratio</th>
<th>Liver</th>
<th>Bursa</th>
<th>Spleen</th>
<th>Lesion Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.100±0.158</td>
<td>0.3500±0.0158a</td>
<td>0.1780±0.0130a</td>
<td>0.00±0.00b</td>
</tr>
<tr>
<td>30</td>
<td>6.140±3.167</td>
<td>0.2420±0.0192c</td>
<td>0.1100±0.0071c</td>
<td>3.60±0.55a</td>
</tr>
<tr>
<td>60</td>
<td>7.380±0.192</td>
<td>0.2960±0.0114b</td>
<td>0.1360±0.0089b</td>
<td>3.40±0.55a</td>
</tr>
<tr>
<td>120</td>
<td>6.980±0.192</td>
<td>0.3160±0.014b</td>
<td>0.1500±0.0100b</td>
<td>3.40±0.54a</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>6.650±1.565</td>
<td>0.3010±0.0424</td>
<td>0.1435±0.0268</td>
<td>2.60±1.60</td>
</tr>
<tr>
<td>P value</td>
<td>0.516</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 14: Organ to body weight ratio (Liver, Bursa, Spleen) and gross lesion score (Liver) after treatment of EGCG

<table>
<thead>
<tr>
<th>Organ to body weight ratio</th>
<th>Liver</th>
<th>Bursa</th>
<th>Spleen</th>
<th>Lesion Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.100±0.158c</td>
<td>0.3500±0.0158</td>
<td>0.1780±0.0130a</td>
<td>0.00±0.00b</td>
</tr>
<tr>
<td>30</td>
<td>6.760±0.1673b</td>
<td>0.3300±0.0158</td>
<td>0.1480±0.0130ab</td>
<td>2.60±0.55a</td>
</tr>
<tr>
<td>60</td>
<td>6.860±0.114ab</td>
<td>0.3180±0.0192</td>
<td>0.1340±0.0230b</td>
<td>2.80±0.84a</td>
</tr>
<tr>
<td>120</td>
<td>7.040±0.167a</td>
<td>0.3260±0.0182</td>
<td>0.1480±0.0192ab</td>
<td>3.00±0.71a</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>6.690±0.391</td>
<td>0.3310±0.0199</td>
<td>0.1520±0.0230</td>
<td>2.10±1.37</td>
</tr>
<tr>
<td>P value</td>
<td>0.000</td>
<td>0.057</td>
<td>0.008</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 15: Organ to body weight ratio (Liver, Bursa, Spleen) and gross lesion score (Liver) after treatment of GTE

<table>
<thead>
<tr>
<th>Organ to body weight ratio</th>
<th>Liver</th>
<th>Bursa</th>
<th>Spleen</th>
<th>Lesion Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.100±0.158bc</td>
<td>0.350±0.016</td>
<td>0.17800±0.01303a</td>
<td>0.00±0.00c</td>
</tr>
<tr>
<td>50</td>
<td>6.440±0.114a</td>
<td>0.336±0.021</td>
<td>0.14600±0.0194b</td>
<td>2.20±0.45a</td>
</tr>
<tr>
<td>100</td>
<td>6.060±0.114c</td>
<td>0.350±0.016</td>
<td>0.17600±0.01140a</td>
<td>0.20±0.45b</td>
</tr>
<tr>
<td>150</td>
<td>6.300±0.100ab</td>
<td>0.322±0.019</td>
<td>0.15200±0.01303ab</td>
<td>0.60±0.55b</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>6.225±0.194</td>
<td>0.3395±0.2038</td>
<td>0.16300±0.0198</td>
<td>0.75±0.97</td>
</tr>
<tr>
<td>P value</td>
<td>0.000</td>
<td>0.076</td>
<td>0.005</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Body weight gain:

Body weight gain was also observed in this experiment and the group which was treated with green tea extract showed maximum weight gain overall. But GTE at 100 mg/ml gave highest body weight gain. The weight gain was recorded daily up to 7 days from the day of treatment and average was calculated. Maximum weight gain was observed in GTE followed by EGCG followed by EGC followed by ECG as shown in figure 17.
Figure 17: Body weight gain (14-21 days) of Broiler chickens

**Histopathological lesion scoring**

Histopathological lesion scoring of bird organs including liver, bursa and kidney with EGC, EGCG, ECG at 30, 60 and 120 mg/ml and severity of lesions were found higher in 30 followed by 60 followed by 120mg/ml. In GTE treated group the concentration of 50 mg/ml gave maximum lesion score but 100 mg/ml gave negligible lesion score in liver, bursa and kidney.

On the basis of lesions score severe lymphoid depletion of bursa of Fabricius of bird treated with 30 mg/ml of ECG and Mild lymphoid depletion of bursa of Fabricius of bird treated with 100 mg/ml of GTE was observed as shown in Figure 18 A, B. In case of kidney severe congestion, hemorrhage and cell infiltration was found in the bird treated with 30 mg/ml of ECG but Mild degenerative changes were observed in birds treated with 100 mg/ml of GTE as shown in figure 19 A, B. The birds treated with 30 mg/ml of ECG gave liver mononuclear cell infiltration with sever congestion and hemorrhage and the liver of the birds treated with 100 mg/ml of GTE gave mild lesion as shown in Figure 20 A, B.
**Figure 18 (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 (H and E Stain, 200X)
Figure 19 (A): Severe congestion, hemorrhage and cell infiltration of kidney of the bird treated with 30 mg/ml of ECG (B) Mild degenerative changes in kidney of birds treated with 100 mg/ml of GTE (H and E Stain, 200X)

Figure 20 (A): Liver mononuclear cell infiltration with severe congestion and hemorrhage of the bird treated with 30 mg/ml of ECG (B) liver of the birds treated with 100 mg/ml of GTE with mild lesion
**Figure 21**: Lesion scoring of ECG at 30, 60 and 120 mg/ml

**Figure 22**: Lesion scoring of EGC at 30, 60 and 120 mg/ml
**Figure 23:** Lesion scoring of EGCG at 30, 60 and 120 mg/ml

**Figure 24:** Lesion scoring of GTE at 50, 100 and 150 mg/ml
CHAPTER-5

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).

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). Catechins of green tea and its extract account for different activity including antimicrobial and antiviral activity (Xu et al., 2008; Weber et al., 2003; Ueda et al., 2013) beside these activities green tea also possess antioxidant and anti-inflammatory activity (Song et al., 2005; Dona et al., 2003) and antitumor (Mukthar and Ahmad, 2000). The reported antiviral activity of green tea extract and its polyphenolic compounds against different viruses including adenovirus, influenza virus, HIV, Epstein bar virus, hepatitis C virus (HCV), HBV, herpes simplex virus type 1 and 2, norovirus and enterovirus 71 (Lin et al., 2013; Oh et al., 2013).

IBH-HPS is an emerging, economically important disease of 3-6 week old broiler chickens (Balamurugan and Kataria, 2004). It is characterized by a sudden onset of mortality (20-80%), hydropericardium and friable, swollen livers with necrosis (Balamurugan and Kataria, 2004). Epidemiological investigations have shown fowl adenovirus type 4 (FAdV- 4) as the causative agent (Voss et al., 1996; Mazaheri et al., 1998) with a genomic similarity of the viruses involved in IBH/HPS outbreaks in different geographical areas (Hess et al., 1999).

One of the most important economic goals in the poultry industry is to minimize the losses by infectious diseases by means of effective vaccination (Fingerut et al., 2003). Vaccination against viral diseases in poultry does confer protection due to various factors which lead to vaccine failure. There is
extensive use liver homogenate vaccine against FAdV-4 causative agent of IBH-HPS in broiler which is not effective to prevent the disease.

The present study was planned to develop alternative treatment for the control of IBH-HPS in broiler. Though, due to drug resistance apprehension antiviral medications in animals are not approved (Ilyushina et al., 2005). We studied the antiviral effects of natural compounds, the catechins in green tea against FAdV-4. The effect of GTE and EGCG, EGC, ECG and GTE was evaluated *in-vitro* against FAdV-4. Catechins in green tea and its isolated compound particularly EGCG inhibiting the adenovirus by direct inactivation of virus, inhibit the virus and by inhibiting the adenain a viral protease *in vitro* (Weber et al., 2003). Keeping in view the antiviral activity of green tea and its isolated polyphenolic compounds were use to evaluate the antiviral activity these test compounds in cell culture and these compounds were evaluate *in-vivo* in poultry birds. Vero cell line was used as a host to propagate the FAdV-4 and for the evaluation of antiviral activity *in vitro*. The cell line was maintained according to protocol adapted by (Freshny, 2000). Vero cell line was received in frozen form that was revived, subculture to made monolayer of cells and cryopreserved for future use. The viability and the no of cells were determined. The virus was obtained from the Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan. The procured virus was propagated in monolayer of Vero cell line to obtain the virus stock that was used to infect the monolayer of the cells. According to the literature Vero cell was used to grow adenovirus, blind passage up to the fifth passage was not shown any cytopathic effects (CPEs), in the 7th passage there was appearance of the CPEs in the Vero cells which was coincide with the results observed by the Roy et al, (2001). In cell culture, FAdV-4 causing IBH-HPS in poultry was shown CPEs characterized by the clumping and aggregation of cells like grape cluster that confirmed the adenovirus. Adenovirus of CPEs was very characteristic that a show bunches of grapes by clumping and rounding of the affected cells to similar to regular cluster. The propagated virus on Vero cell line was confirmed
through the AGPT and RPHA test. The virus was confirmed through these tests as FAdV-4 that confirmed virus was used for the antiviral assay (Mansoor et al., 2009). There was clear and consistent CPEs were observed for the propagation of the virus on Vero cells, these founding was coincided with the previous results obtained by Roy et al. (2001).

The plaque forming unit (PFU/ml) was calculated through the plaque assay. The assay depend upon the uptake of the neutral red dye by the dead cells having the distinct advantage over the crystal violet a non vital dye, the formalin fixed cells was stored for the large time and does not precipitate in water. Plaque assay results were shown that the dilution of $10^{-5}$ was shown a 500 PFU/ml of the virus.

The PFU/ml was calculated to infect the monolayer of the cell for the plaque inhibition assay to evaluate the antiviral effects of tested compounds and the plaques were calculated with the control without any test compounds. Plaque formation assay was a indirect test to measure the replication of adenovirus through the examination of CPE was a relatively easy and inexpensive (Gordon et al., 1991). Cell culture studies in vitro provide the preliminary data that was for the in-vivo trial which is the useful means for the screening of the antiviral agents (Wang et al., 2010). The cell culture methods was used for the screening of the antiviral compounds in vitro in the laboratory through different methods by plaque reduction assay, virus reduction assay with different format virus was harvested from cell by and calculated the virus yield to determine the antiviral effects of different antiviral compound (Song et al., 2005).

To evaluate the cytotoxicity of the test compounds MTT assay was performed that was based on the measurement mitochondrial metabolic rate using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to indirectly reflect viable cell numbers has been widely applied (Thangapazham et al., 2007). The MTT assay was used for the cell proliferation and cytotoxicity. The MTT assay was more sensitive and less time consuming method for the
viability of cell as compare to other method that was very time consuming and accurate by trypan blue dye exclusion assay.

In the present project MTT assay was performed to evaluate the inhibition of normal cell without infection with the virus, the compound was tested in cell culture through the MTT assay to evaluate the viability of the cells. MTT assay was calculated the tolerable concentration of the EGCG, EGC, ECG and GTE that was used in antiviral assay. The maximum cell inhibition was observed at 150 µg, 200 µg and 250 µg of GTE and 120 µM, 180 µM, 240 µM of EGCG, EGC ECG was shown the inhibition at dose dependent manner respectively. The compounds were selected for antiviral assay that was less toxic to the cell having 30 µM, 60 µM and 120 µM of EGCG, EGC and ECG and in case of GTE 50 µg, 100 µg and 150 µg was selected. The green tea catechins, particularly EGCG reported to be more toxic to cancer cells than the normal cells (Morre et al., 2000).

Antiviral activity was observed through plaque inhibition assay and the EC$_{50}$ was calculated. The results was shown the in our study that ECG in plaque reduction assay the reduced the plaque number was maximum at 120 µM concentration up to 20% inhibition and the most effective antiviral activity of GTE was observed in plaque reduction assay at 100 µM followed by 150 µM and 50 µM as compare to control. The EC$_{50}$ of GTE 34.80 and CC$_{50}$ was 100.23 with a SI 3.165 that results were shown that high SI showing maximum antiviral activity with minimum CC$_{50}$ according to the guide line for the evaluation of the antiviral drugs. These results similar to the results of Imanishi et al, (2002) observed that influenza virus infectivity was inhibit by GTE and EGC through the acidification of ELS that inhibit the growth of influenza A and B virus in MDCK cells and GTE inhibit the V-H pump. Weber et al, (2003) observed that antiviral effects of GTE and EGCG against the adenovirus which inhibits the cellular process, so the virus replication depends on these cellular processes and the EGCG not only inhibit the cellular processes but also promote the release of virus from the cell. The results of plaque inhibition assay in our study similar to results was observed by Song et al,
which observed that the polyphenolic mixture showed more efficient results against the influenza virus as compared to the single compound. In this study EGCG, ECG was more effective as compared EGC which was 10 fold more effective against influenza virus through plaque inhibition assay. The mechanism of inhibition was by direct contact with the virus and membrane fusion was interfere by the acidification of the endosome and at higher concentration of EGCG and ECG which inhibit the synthesis of viral RNA because the catechins the back bone of green tea concerned with the DNA polymerase.

In the virus reduction assay, which was depends on the virus infectious particle on time course basis, after different time interval the virus was harvested and was subjected to plaque assay to calculate the virus yield through the titration of the virus on Vero cells to observe the antiviral effects against the FAdV.

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. The maximum virus yield reduction was in EGCG at 120 µM of EGCG which was observed at 36 hours of post infection with the control followed by 60 µM and 30 µM. 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 µM 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 hours post infection followed by 150 µg and 50 µg. GTE was shown maximum inhibition of virus on plaque assay after harvesting the virus at various times intervals and reduce the virus yield at dose depend manner and there antiviral effects was at all stages of the infectious cycle. These results of virus yield reduction assay was coincide with the finding of Song et al, (2005) observed that isolated compounds EGCG was the most effective antiviral compounds against influenza, and ECG was the less effective compound among the tested compound. They observed that polyphenolic mixture was very effective at all stages of the virus infectious cycle. The complete inhibition was
observed 120 µM of EGCG against influenza virus. The calculated EC$_{50}$ and CC$_{50}$ was shown that GTE EC$_{50}$ was 32.80 µg and CC$_{50}$ was 100.23 µg/ml with SI 3.165, these results were according to Oh et al., (2013) that observed the green tea against the feline calicivirus (FVS) having EC$_{50}$ values 0.13 mg/ml meaning that anti-FCV activity with lowest concentration and CC$_{50}$ 18.57 of GTE was the highest of the test compounds, means that it has strong antiviral activity with low toxicity. Our experiment finding suggested that GTE was strong antiviral activity against FAdV-4 lowest EC$_{50}$ and highest CC$_{50}$ with higher SI 3.165 was observed. In vitro study showed that the GTE was more efficient than other compounds which were confirmed in other finding due the presence of other active components of GTE (Xu et al., 2008).

In vitro findings were expanded to evaluate the antiviral effect of these compounds In-vivo experiment, the birds in all the groups were challenged with FAdV-4 at 15$^{th}$ day and at the same day treatment with different doses of test compounds were giving for the 7 days. The mortality and morbidity were observed for the whole period of the treatment. The gross lesions were higher in infected untreated control that includes the lesions in kidney, liver and the spleen of birds. The GTE treated group was shown no gross and pathological lesions having no symptom of hydropericardium and there was no enlargement of liver that followed by EGCG, EGC and ECG with mild lesions were observed in these treated groups. These finding of our results were according to the finding of Barbour et al., (2007), against influenza virus challenge in chicken, following parameters were improved by giving Epican Forte ® absence of rales and gross lesions including tracheitis and enteritis. The tea polyphenolic compounds have antiviral activity against viruses which was already demonstrated in different studies and against influenza virus in-vivo (Lee et al., 2012). The protection of broiler chicks observed in GTE treated group was 90% at 100 mg/ml followed by 150 mg/ml and 50 mg/ml followed by EGCG, EGC, and ECG that protected the birds maximum at 120 mg/ml respectively following challenge. The body weight gain in different groups treated with GTE,
EGCG, EGC and ECG were observed during the treatment groups, the maximum weight gain was observed in GTE treated birds compared with the control groups at 100 mg/ml that was coincide 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. Organ to body weight ratio was observed all the treated groups compared to control, ECG gave statistically significant difference of organ to body weight ratio at all concentration. In EGC treated group liver to body weight ratio was non significant but spleen and bursa weight ratio were statistically different at all concentration. In EGCG treated group spleen and liver weight ratio were significant statistically as compared to control but bursa to body weight ratio was non significant. In GTE treated group liver to body weight ratio at 100 mg/ml was non significant statistically in comparison with control and bursa to body weight ratio had non significant difference at all concentration but spleen to body ratio was non significant at 100 mg/ml concentration of GTE. From above all discussion it was concluded that GTE at 100 mg/ml concentration gave maximum protection against challenge with FAdV-4.

Upon histopathology maximum lesion score was observed at concentration of 30 followed 60 followed 120 mg/ml in case of liver, bursa and kidney in EGCG, EGC and ECG. In case of EGC statistically significant lesion score was observed at all concentrations. In case of EGCG maximum mean lesion score was observed at 120 mg/ml followed by 60 mg/ml followed by 30 mg/ml. In GTE treated group the concentration of 50 mg/ml gave maximum lesion score and 100 mg/ml gave negligible lesion score. So it was concluded that the GTE at 100 mg/ml concentration gave minimum lesions observed in histopathological examination. The green tea extract was maintained the original appurtenance of tested tissues which was accordance with the finding of Gawish et al, (2010) which was observed the green tea extract against nicotine toxicity that restore the original appreance of testicular tissue. Regarding it affects on liver in our study GTE was shown minimum gross,
pathological and histopathological lesions which was agreement with the finding of Avwioro et al, (2010) observed that GTE had no adverse effects on the histology and biomarker of liver.
CHAPTER-6

SUMMARY

Green tea from (*Camellia sinensis*) has a unique nutritional values which have diverse activity for the benefits of human and animals. Green tea has various biological and pharmacological activities including anticancer, antioxidant, antibacterial and antiviral effects. The project was designed to explore the antiviral effect green tea against fowl adenovirus type 4 (FAdV-4), a virus cause economically important disease in poultry which cause heavy economic losses to the poultry industry in Pakistan. Vero cells were established and subculture and monolayer was prepared to infect with the virus to observe the antiviral activity against the FAdV. The FAdV-4 was propagated in Vero cell. The plaque of the virus was calculated to infect the monolayer of cell. To evaluate the antiviral effect of green tea extract, EGCG, EGC and GTE in cell culture to access the dose of these compounds for in-vivo trial in poultry birds. The CC50 of EGCG was evaluated at different concentration from 60-240 µM to check the viability of cell by MTT assay, the maximum inhibition was observed at 180 and 240 µM of EGCG, so the tolerable concentration was 30, 60 and 120 were observed in MTT assay. So that the same results were obtained in case of EGC and ECG in CC50 study. However, in case of GTE, there were more tolerance of cells and at concentration of 250 µg there was inhibition of more than 50% was observed, From the initial toxicity study, so selected dose for the in vitro antiviral study was EGCG, EGC and ECG was 30, 60 and 120 µM respectively and for GTE selected dose was 50, 100 and 150 µg. Plaque inhibition assay of EGCG, the maximum inhibition was observed at 120 µM of this compound which was 36.8421±2.10526 followed by 60 and 30 µM. EGC inhibit the maximum plaque at 120 µM 28.7719±3.21584 followed by 30 and 60 µM, ECG was also showed maximum inhibition at the same concentration as shown in EGCG and EGC. GTE maximum inhibition was observed at 100 µg which was 77.7583±2.90658% followed by concentration of 150 µg and 60 µg of green tea extract. Virus yield reduction assay, observed the effects of green tea extract and isolated compounds on virus yield at different times of post
infection of Vero cells. The cells were infected with FAdV-4 at 0.1 multiply of infection and different concentration of test compounds was added in culture medium. Harvest the virus at various time intervals 8, 24 and 36 hours after virus infection. This harvested virus was subjected to plaque assay for reduction in virus yield. Virus yield in EGCG treated cells was observed, which was maximally reduced at 36 hours of post infection with the concentration of 120 µM followed by 60 and 30 µM and other tested concentration was showing same reduction in virus yield at different times interval of post infection. In case of EGC complete reduction in virus yield was observed at 120 µM at 36 hours of post infection and at 60 µM of EGC showed maximum reduction in virus yield at 36 hours of post infection followed by 30 µM of EGC. ECG also showed complete reduction in virus yield at 24 and 36 hours at 120 µM concentration and same was observed in ECG as was observed in EGC at 60 and 30 µM this compound. GTE maximum reduction was observed at 100 µg at 24 and 36 hours of post infection which was followed by 120 µg and 50 µg of green tea extract. EC50 and CC50 of these tested compounds were also calculated. EC50 of EGCG was 207.12 and CC50 was 59.56 and the SI of this compound was 0.28, EGC was 241.70 and CC50 was 78.88 and the SI of this compound was 0.32 and ECG was 392.52 and CC50 was 34.05 and the SI of this compound was 0.089. GTE was shown EC50 34.80 and CC50 109.23 and SI of this 3.135. So, Green tea was high SI which maximum antiviral efficacy against FAdV \textit{in-vitro}.

The compounds was shown antiviral activity against FAdV were evaluated \textit{in-vivo} in poultry birds. The results of this study have shown that maximum survival rate in GTE treated was observed at 100 mg followed by EGCG, EGC and ECG. Maximum organ to body weight ratio was also observed in GTE treated group and gross and histopathological lesion scoring were observed in GTE treated group. GTE has been proved to be an alternative treatment of FAdV infection in poultry in a situation where vaccine failure occurred, vaccine could not dilevered due to any problem e.g., disease, vaccine availability, or vaccine is not routinely used in an area.
CHAPTER-7

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