Anti-avian influenza virus H_9N_2 activity of aqueous extracts of *Zingiber officinalis* (Ginger) and *Allium sativum* (Garlic) in chick embryos

Amir Rasool[1], Muti-ur-Rehman Khan[1], Muhammad Asad Ali[2], Aftab Ahmad Anjum[2], Ishtiaq Ahmed[1], Asim Aslam[1], Ghulam Mustafa[1], Saima Masood[3], Muhammad Amjad Ali[4] and Muhammad Nawaz[2]

[1]Department of Pathology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[2]Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[3]Department of Anatomy and Histology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[4]Faculty of Veterinary Sciences, Bahauddin Zakariya University, Multan, Pakistan

Abstract: In the present study, anti-Avian influenza virus H_9N_2 activity of aqueous extracts (5, 10, 15, 20, 25%) of *Zingiber officinalis* and *Allium sativum* was evaluated. Embryo-toxicity was evaluated by histopathological scoring of Chorio-allantoic membrane of chick embryos. Cytotoxicity of extracts was determined by MTT assay on Vero cells. Aqueous extract of ginger had antiviral activity at 10, 15, 20 and 25% while garlic had activity at 15, 20 and 25%. Histopathological scoring of chorio-allantoic membrane for aqueous extracts (5, 10, 15, 20, 25%) of ginger (0.66±0.57, 1.33±0.57, 1.66±0.57, 2.66±0.57, 3.66±0.57, respectively) and garlic (1.00±0.00, 1.33±0.57, 2.00±0.00, 2.33±0.57, 3.66±0.57, respectively) was concentration dependant. MTT assay revealed cytotoxicity of both plants was also concentration dependent. Extracts of ginger (5, 10, 15, 20, 25%) had lower cytotoxicity (71, 59, 28, 22, 0 % cell survival, respectively) as compared to garlic (61, 36. 20, 11, 3% cell survival, respectively). Overall results revealed that concentration of aqueous extract of ginger (10%), showing antiviral activity against H_9N_2, was less toxic to vero cells (> 50% cell survival). It is insinuated that ginger may have anti- Avian influenza virus H_9N_2 potential and its active compounds needs further investigations.

Keywords: Avian Influenza virus, *Zingiber officinalis*, *Allium sativum*, antiviral, cytotoxic, chick embryo, MTT assay.

INTRODUCTION

Avian influenza virus causes serious disease in a wide variety of birds, including wild ducks, gulls, shorebirds and poultry. It causes problems of respiratory tract of poultry leading to lower yield, or a rapidly fatal systemic disease known as highly pathogenic avian influenza (Subtain *et al.,* 2011). Supportive care and antibiotic treatment are generally employed to reduce the effects of concurrent bacterial infections as an aid in recovery of poultry and other birds from highly pathogenic avian influenza (Swayne, 2009).

Plants are source of innumerable chemical compounds used in medicine. There is an ever expanding interest in use of natural products to prevent viral infections. Many of the modern drugs have been derived from plants (Jassim and Naji, 2003). Many of the research groups throughout the world have shifted their focus on identification of benefits of plant extracts and their utilization as valid alternatives of antimicrobials (Kamel, 2000). Different organic and inorganic solvents are used to extract active compounds of plants followed by their purification by chromatographic techniques (Wheeler, 1993).

*Corresponding author: e-mail: asad.ali@uvas.edu.pk

Different researchers, working on phytomedicines, have reported antiviral activity of ginger and garlic against different viruses causing infections in humans and poultry birds (Chang *et al*., 2013; Pushpa *et al*., 2013; Augusti *et al*., 2010; Dieumou *et al.,* 2009; Koch *et al*., 2008; Nagai, 1973).

In present study, anti-Avian influenza virus H_9N_2 activity of *Zingiber officinalis* and *Allium sativum* was evaluated. Embryo-toxicity and cytotoxicity of *Zingiber officinalis* and *Allium sativum* were also determined by egg inoculation and MTT assay, respectively.

MATERIALS AND METHODS

Dried *Zingiber officinalis* rhizomes and *Allium sativum* cloves (10g, each) were grinded, soaked in 100ml distilled water at room temperature for 24 h and extracts were collected by filtration through filter paper (0.22µm). The filtrates were dried incubating at 37ºC in incubator to obtain the aqueous extract (AE) (Machana *et al*., 2012). Five concentrations (5, 10, 15, 20 and 25%) of each dried plant extracts were prepared in distilled water.

The virus was mixed with each concentration (5, 10, 15, 20, 25%) of each plant extract to make an extract/viral suspension. The mixtures were inoculated in 9-day old embryonated chick eggs. Each egg was inoculated with

0.1ml of the inoculum and five eggs were inoculated with each concentration of the extract/virus suspension, while extract only (without virus) and virus only (without extract) were used as negative and positive controls, respectively. After 48h of incubation, the embryos were chilled and their allantoic fluids harvested. The virus presence in allantoic fluid was checked by rapid HA (haemagglutination) test using 2% chicken RBCs suspension (Allan and Gough, 1974).

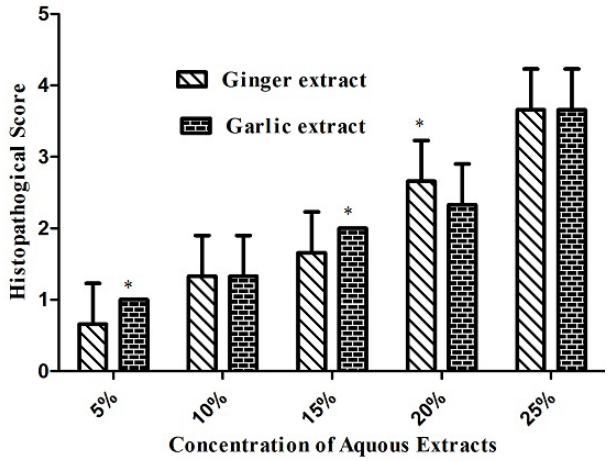

Fig. 1: Mean Histological scoring of Chorio-allantoic membranes of chick embryos inoculated with different concentrations of ginger and garlic aqueous extracts.

Chorio-allantoic membrane of each egg was observed for histo-pathological lesions and scored to determine the effect of the extracts on pathogenicity of avian influenza virus H_9N_2 (Sulaiman *et al*., 2011; Lierz *et al*., 2007). Epithelial hyperplasia, vacuolar degeneration, inclusion corpuscles, inflammation and congestion or edema were studied and lesions scored as: Severe lesions (4), Moderate + severe lesions (3), Moderate lesions (2), Mild lesions (1) and No lesions (0).

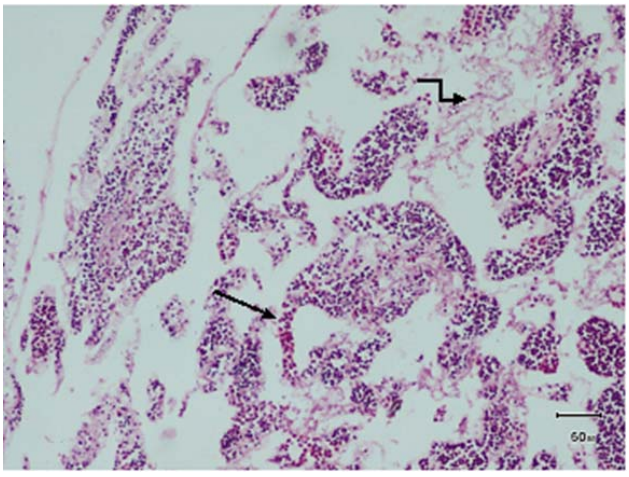

Fig. 2: Arrow: Mild changes (Congestion (Accumulation of RBC's) Zigzag Arrow: Degeneration (Breakdown of cells).

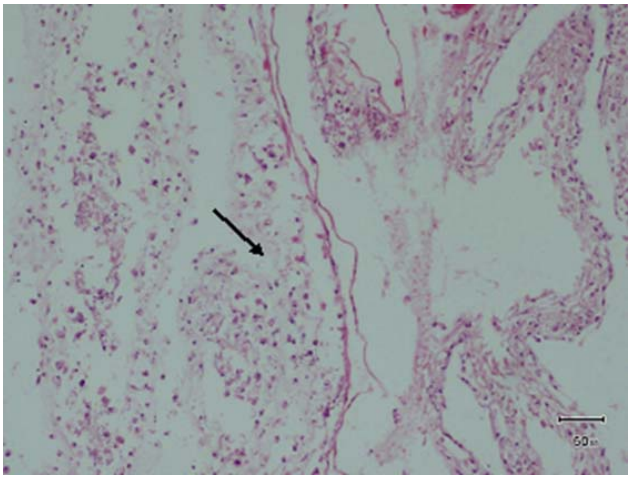

Fig. 3: Arrow: Very severe necrosis (Cell death and degeneration).

Cytotoxicity of extracts was determined by MTT [3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay (Celis, 1998). The cells were cultured using M-199 medium (Freshney, 2010). The experimental group consisted of media, different dilutions of each plant extract (200ug/ml, of each concentration) and MTT dye, whereas the control group comprised of media only. These experimental and control preparations were added in separate wells. Each concentration was tested in three wells. The plate was incubated at 37˚C for 48 hours. And an optical density (OD) value of each well was recorded at 570nm.

STATISTICAL ANALYSIS

Collected data was analyzed by using One Way ANNOVA and DMRT (applied to the data by using Statistical Package for Social Sciences).

RESULTS

Antiviral activities of aqueous extracts of ginger and garlic are presented in table 1. Histopathological scoring of changes on chorio-allantoic membrane of chicken embryo is given in fig 1. Representative histopathological changes are shown in fig. 2 and 3. Cytotoxicity of ginger and garlic extracts as determined by MTT assay is presented in table 2

DISSCUSSION

Anti-viral activity, histopathological effects on chorio-allantoic membrane of chick embryos and cytotoxicity of *Zingiber officinalis* (Ginger) and *Allium sativum* (Garlic) plants were evaluated. Different concentrations of aqueous extracts of garlic cloves and ginger rhizome were evaluated for their antiviral activity against Avian influenza virus H9N2 by inoculating mixture of the

extract and the virus into 9 - day old chick embryos. It was observed that higher concentrations of garlic (15% and above) had anti-viral effect as compared to ginger (10% and above) as indicated in table 1.

Table 1: Antiviral activity of different dilutions of garlic and ginger extracts against Avian influenza virus H_9N_2

Dilution of extract	Antiviral effect of	
	Ginger	Garlic
5%	-	-
10%	+	-
15%	+	+
20%	+	+
25%	+	+

Different researchers working on phytomedicines have reported ginger and garlic extracts antiviral activity against different viruses causing infections in humans and poultry birds (Pushpa *et al*., 2013; Augusti *et al*., 2010). Antiviral activity of ginger was proved against various viruses (Ali *et al*., 2008; Koch *et al*., 2008; Schnitzler *et al*., 2007; Sookkongwaree *et al*., 2006). Chang *et al*., (2013) demonstrated antiviral effect of fresh ginger against Human respiratory syncytial virus on HEp2 and A549 cell line. Denyer *et al*. (1994) reported anti-HRS and anti-rhinoviral activity. Nagai (1973) reported anti Influenza virus activity of garlic. Later on, researchers reported antiviral activity of garlic (Haris *et al*., 2001; Ankri and Mirelman, 1999). For instance, in an *in vitro* study, garlic extract exerted a dose-dependent antiviral effect against human cytomegalovirus. Other *in vitro* studies documented a virucidal activity of garlic extract against herpes simplex types 1 and 2 virus, parainfluenza virus type 3, vaccinia virus, vesicular stomatitis virus and human rhinovirus type 2 (Mehrbod *et al*., 2009; Vahabpour-Roudsari *et al*., 2007).

Highest mean histopathological scoring (3.66±0.57) of chorio-allantoic membranes was recorded for chick embryos inoculated with 25% garlic aqueous extract followed by 20, 15, 10 and 5% as 2.33±0.57, 2.00±0.00, 1.33±0.57 and 1.00±0.00, respectively (fig. 1). Highest mean histopathological scoring (3.66±0.57) on chorio-allantoic membranes was recorded for chick embryos inoculated with 25% ginger aqueous extract followed by 20, 15, 10 and 5% as 2.66±0.57, 1.66±0.57, 1.33±0.57 and 0.66±0.57, respectively (fig. 1). Vilela *et al*. (2011) also conducted histopathological evaluation of green propolis ethanolic extracts on chorio-allantoic membrane of chick embryos. The results of the study were in accordance to the present study that the decrease in extract concentration reduced histopathological scoring on the membrane. Moreover, the time of incubation was directly proportional to the histopathological scores on the membrane. Representative plates showing histological lesions are presented in figs. 2 and 3.

Five concentrations (5, 10, 15, 20, 25%) of each dried plant extract were evaluated for cytotoxicity by MTT [3-(4, 5-dimethyl thiazole-2-yl)-2, 5-diphenyltetrazolium bromide] assay in triplicates. Cell survival percentage was recorded 71, 59, 28, 22, 0% for ginger extract and 61, 36, 20, 11 and 3% for garlic extract for each concentration (5, 10, 15, 20, 25%, respectively).

It was observed that concentration of aqueous extracts was inversely related to cell survival percentage. These findings are in agreement to those of Al-Niamey (2013) who reported 70% cell survival at the lowest concentration (0.390µg/ml). Shrivastava and Ganesh (2010) reported similar results in the study on cell viability using 50mg and 100mg concentrations of Garlic extracts. Furthermore, it was found that South Indian garlic extract was more potent in minimizing the cell viability up to 47% compared to ginger.

CONCLUSION

It is insinuated that ginger may have anti- Avian influenza virus H_9N_2 potential and its active compounds needs further investigations.

Table 2: Mean OD values of MTT assay at 570 nm for different concentrations of garlic and ginger aqueous extracts

Aqueous extract Percentage	Mean OD value (570nm)	Cell survival percentage
Garlic 25	0.1037	03
Garlic 20	0.1293	11
Garlic 15	0.1547	20
Garlic 10	0.2033	36
Garlic 05	0.2853	61
Ginger 25	0.0920	00
Ginger 20	0.1620	22
Ginger 15	0.1803	28
Ginger 10	0.2737	59
Ginger 05	0.3093	71
+ve (Cell culture medium)	0.3030	00
-ve (20% DMSO)	0.0947	00

REFERENCES

Ali BH, Blunden G, Tanira MO and Andnemmar A (2008). Some phytochemical, pharmacological and toxicological properties of ginger (Zingiberofficinale roscoe): A review of recent research. *Food Chem. Toxicol.*, **46**: 409-420.

Allan WH and Gough E (1974). A standard haemaglutination inhibition test for Newcastle disease: comparison of macro and micro-methods. *Vet. Record*, **95**: 120-123.

Al-Niaame E (2013). A study of antibacterial activity and histological effects of *Eruca sativa* (L.) seeds on male mice. *Al-Mustansiriyah J. Sci.*, **24**(1): 9-22.

Ankri S and Mirelman D (1999). Antimicrobial properties of allicin from garlic. *Microbes Infect.*, **1**(2): 125-129.

Augusti KT, Jose R and Augustine P (2010). Antiviral, anti-inflammatory and related effects of a food supplement made of garlic, ginger and black pepper. *Ind. J. Clin. Biochem.*, **25**(2): 217-218.

Celis JE (1998). Review from A Laboratory Handbook of Cell Biology. Second Edition MTT. *Cell Proliferation Assay*, **1**: 16-18.

Chang JS, Wang KC, Yeh CF, Shieh DE and Chiang LC (2013). Fresh ginger (*Zingiber officinale*) has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines. *J. Ethnopharmacol.*, **145**(1): 146-151.

Denyer CV, Jackson P, Loakes DM, Ellis MR, Young DA. 1994. Isolation of antirhinoviral sesquiterpenes from ginger (*Zingiber officinale*). *J. Nat. Prod.*, **57**: 658-662.

Dieumou FE, Teguia A, Kuiate JR, Tamokou JD, Fonge NB and Dongmo MC (2009). Effects of ginger (zingiberofficinale) and garlic (allium sativum) essential oils on growth performance and gut microbial population of broiler chickens. *Livestock Research for Rural Development*, **21**: 25-34.

Freshney RI (2010). Culture of Animal Cells, A Manual of Basic Technique. 6th ed. Hoboken (New Jersey): John Willey & sons (U.S.A). Pp: 314.

Harris J, Plummer S and Lloyd D (2001). Antigiardial drugs. *Appl. Microbiol. Biot.*, **57**: 614-619.

Jassim SAA and Naji MA (2003). Novel antiviral agents: a medicinal plant perspective. The Society for Applied Microbiology. *J. Appl. Microbiol.*, **95**: 412-427.

Kamel C (2000). A Novel Look at a Classic Approach of Plant Extracts. Feed Mix, pp.19-21.

Koch S, Reichling J, Schneele J and Schnitzler P (2008). Inhibitory effect of essential oils against herpes simplex virus type 2. *Phytomed.*, **15**: 71-78.

Lierz M, Bergmann V, Isa G, Claus P, Lueschow D, Mwanzia J, Prusas C and Hafez H (2007). Avipoxvirus infection in a collection of captive stone curlews (*Burhinus oedicnemus*). *J. Avian Med. Surg.*, **21**(1): 50-55.

Machana S, Weerapreeyakul N, Barusrux S, Thumanu K and Tanthanuch W (2012). Synergistic anticancer effect of the extracts from *Polyalthia evecta* caused apoptosis in human hepatoma (HepG2) cells. *Asian Pac. J. Trop. Biomed.*, **2**(8): 589-596.

Mehrbod P, Amini E and Tavassoti-Kheiri M (2009). Antiviral Activity of Garlic Extract on Influenza Virus. *Iranian J. Virol.*, **3**(1): 19-23.

Nagai K (1973). Experimental studies on the preventive effect of garlic extract against infection with influenza virus. *Japanese J. Inf. Dis.*, **47**: 321.

Pushpa R, Nishant R, Navin K and Punkaj G (2013). Antiviral potential of medicinal plants; An overview. *Int. Res. J. Pharm.*, **4**(6): 8-16.

Schnitzler P, Koch C and Reichling J (2007). Susceptibility of drug-resistant clinical herpes simplex virus type 1 strains to essential oils of ginger, thyme, hyssop, and sandalwood. *Antimicrob Agents Chemother.*, **51**: 1859-1862.

Shrivastava S and Ganesh N (2010). Tumor inhibition and Cytotoxicity assay by aqueous extract of onion (Allium cepa) & Garlic (Allium sativum): An *in vitro* analysis. *Int. J. Phytomed.*, **2**: 80-84.

Sookkongwaree K, Geitmann M, Roengsumran S, Petsom A and Danielson UH (2006). Inhibition of viral proteases by *Zingiberaceae* extracts and flavones isolated from *Kaempferia parviflora. Pharm*., **61**: 717-721.

Subtain SM, Chaudhry ZI, Anjum AA, Maqbool A and Sadique U (2011). Study on Pathogenesis of Low Pathogenic Avian Influenza Virus H9 in Broiler Chickens. *Pak. J. Zool.*, **43**(5): 999-1008.

Sulaiman LK, Oladele OA, Shittu IA, Emikpe BO, Oladokun AT and Meseko CA (2011). *In vivo* evaluation of the antiviral activity of methanolic root-bark extract of the African Baobab (*Adansonia digitata* Linn). *African J. Biotech.*, **10**(20): 4256-4258.

Swayne, D.E. 2009.Avian influenza vaccines and therapies for poultry. *Comp. Immun. Microbiol. Infect. Dis.*, **32**: 351-363.

Vahabpour-Roudsari R, Shamsi Shahrabadi M, Monavari SH and Sajjadi SE (2007). Evaluation of potential antiviral activity of hydroalcoholic extract of Lemon Balm L. against Herpes Simplex Virus type-I. *Iranian J. Virol.*, **1**: 28-32.

Vilela CO, Fischer G, De-Castro CC, Nunes CF, Hübner SO, Raffi MB, Salles SE, Anciuti MA and Vargas GD (2011). Virucidal activity of green propolis against avipoxvirus in chorioallantoic membrane of embryonated chicken eggs. *African J. Microbiol. Res.*, **5**(9): 1075-1082.

Wheeler GE (1993). Use of Herbal Supplements to Reduce the Effect of Stress. Proceedings World Conference on Medicinal and Aromatic Plants, Tiberius, Israel.