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Comparative Efficacy of Various Antiviral Agents against Avian Influenza Virus (Type H7N3/Pakistan/2003)

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Abstract.- Highly pathogenic avian influenza (H7 type) is of greater importance due to its economic impact and public health significance. Drugs available to vanish this pathogens are very limited and having high prices. Therefore a study was planed to check the comparative efficacy of the Herbs' extract as compared to the standard available drug in the market. There were used three different concentrations (2%, 4% and 8%) of each of amantadine HCl, extracts of fresh leaves of opuntia herb, papaya and dried powdered leaves of green tea in distilled water and were evaluated for their antiviral activity, by injecting through chorioallantoic sac route into 10 days old live embryonated broiler chicken eggs. Group A is negative control with no haemagglutination activity and group B as positive control with positive Haemagglutination activity. For each drug to be tested, 40 eggs were assigned to 8 groups (*i.e.* A, B, C1, C2, C3, D1, D2, D3) with 5 eggs in each group. Embryonated eggs in each of C1, C2 and C3 subgroups were treated only with 3 different concentrations of each compound to evaluate toxicity respectively. Whereas embryonated eggs of SUB-GROUPS D1, D2 and D3 were treated with suspensions of three different concentrations of each compound with 4HA titer of Avian Influenza Virus (AIV) type H7N3/Pakistan/2003, to check antiviral efficacy respectively. Positive and negative controls were also run side by side under similar experimental conditions. In case of amantadine HCl out of 50, 500 and 1000 µg/ml concentrations, only 500ug/ml was found to be an ideal concentration, as in addition to stop virus growth it also did not kill the embryos. In case of *Opuntia dellinii* all the 3 concentrations used were not toxic for embryos, but antiviral effect was observed only at 4g and 8g/100ml concentrations. Green tea extract was found to be effective against AIV only at 8g/100ml concentration with no damage to chick embryos. Papaya leaves extract as a whole failed to check virus replication at all the three concentrations used in this experiment *i.e.* 2, 4 and 8g/100ml. All these dose levels were not lethal for chick embryos.

Keywords: Avian influenza virus, herbal extracts, embryonated eggs, influenza virus type H7N3, opuntia dellini.

INTRODUCTION

There is increasing concern that highly pathogenic avian influenza A viruses of the H7 haemagglutinin (HA) pose a pandemic threat to human. Early in 2003, an H7N7 highly pathogenic avian influenza virus caused a poultry outbreak that spread from Netherlands to Germany and Belgium. In Netherlands, this virus was detected in 89 human who handled affected poultry, including three cases of their family members (Fouchier *et al*., 2005; Shahzad *et al*., 2007). In February 2004, H7N3 influenza virus was also isolated from humans in Canada, although no deaths were reported (Lam *et al*., 2008). Influenza virus is an RNA virus of the family Orthomyxoviridae. The virion nucleus contains nucleocapsid that consists of the viral genome, nucleoprotein (NP) and the complex RNA-transcriptase (Ciampor *et al*., 1998). Up to date there are two classes of anti-influenza agents (i) NA inhibitors, oseltamivir, protecting the release and progeny of virion, and (ii) adamantine derivatives, amantadine and rimantadine, preventing the proton transfer in M2 ion channels (DeClercq, 2006). Among the herbs, soyabean and opuntia species are also claimed to have antiviral activity against influenza virus. These herbal plants are supposed to have saponin I and saponin II responsible for their

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antiviral activity (Ahmed *et al*., 1996).

Green tea catechin has been found to have many unique antimicrobiological activities such as antibacterial, antiviral, antifungal and antitoxic effects (Michi *et al*., 2002). Three polyphenolic catechins, epigallocatechin (EGC) (Ciampor *et al*., 1998), epicatechin-3-O-gallate (Wilson and Von Itzstein, 2003) and epigallocatechin-3-O-gallate (EGCG) (Moscona, 2005) were isolated from Chinese green tea (*Camellia sinensis*) and demonstrated a new class of human immuno-deficiency virus-reverse transcriptase (HIV-RT) inhibitor (Chang *et al*., 1993). *Carica papaya* seeds contain anti-bacterial activity that inhibits growth of Gram-positive and Gram-negative organisms. Observed activity was independent of stage of fruit maturity. *Carica papaya* has antibacterial effects that could be useful in treating chronic skin ulcers to promote healing (Dawkins *et al*., 2003). Some phytonutrients in green papaya help to restore proper intestinal flora and have vermifugal, amoebicidal, and antibacterial properties (Quisumbing, 1951).

The highly virulent influenza A viruses which may produce acute clinical disease in chickens and turkeys have been found associated only with the H5 and H7 subtypes (Swayne and Halvorson, 2003). Continuing outbreaks of avian influenza in Asian countries have induced a great loss to poultry industry. Although there is no report of transmission of this virus to human it is still considered to have a great public health significance. Mutations can cause small changes in the hemagglutinin and neuraminidase antigens on the surface of the virus. Different types of antiviral drugs are in use now a days in the world. Generally in Asia and especially in Pakistan, due to poor health care setups and socio-cultural reasons people are more likely to take herbal medicines. Hence an investigation to check the efficacy of the herbs extracts which are supposed to have antiviral activity in comparison to commercially available antiviral drugs.

MATERIALS AND METHODS

Antiviral agents

Different antiviral agents tested for their antiviral activity along with their concentrations used in this experiment are given in Table I.

Virus

AIV type H7 was cultivated in ten 10-day-old embryonated chicken eggs by injecting 0.2 ml of stock viral suspension in each egg through the allantoic sac route. After 48 h of incubation, the embryos were chilled and their allantoic fluids harvested.

Table I. - Different concentrations of antiviral agents to be tested along with plant part used to prepare the extract.

Antiviral agent(s)	Concentrations used		
	Low (Lc)	Medium (Mc)	High (Hc)
Amantadine HCl (Virofral)	50 µg/ml	500 µg/ml	1000 µg/ml
Extract of opuntia herb (fresh leaves of *Opuntia dellinii*)	2g/100 ml	4g/100 ml	8g/100 ml
Green tea solution (dried, powdered leaves of *Camellia sinensis*)	2g/100 ml	4mg/100 ml	8g/100 ml
Extract of papaya leaves (fresh leaves of *Carica papaya*)	2g/100 ml	4g/100 ml	8g/100 ml

The virus presence in allantoic fluid was checked by rapid HA (haemagglutination) test (Allan and Gough, 1974) using 5% RBCs suspension. The virus was diluted appropriately in normal saline solution to obtain 4 HA units of the test virus.

Herbal extracts

Extracts of opuntia herb and papaya leaves were obtained by soaking 8 g of fresh leaves of the respective plant in 100 ml of distilled water for 24 h followed by the grinding of the mixture in a tissue grinder. The extract was filtered by using Millipore single use syringe filters 0.22 µm pore size, centrifuged at 1500 rpm for 5 min, and then reconstituted as 8%, 4% and 2% solutions (Taylor *et al*., 1996). In the case of green tea, 8g of dried, powered leaves (Chengdu Waggott Pharmaceutical Co.) were soaked in hot water for 15 min and then filtered. The filtrate was reconstituted as 8%, 4% and 2% solutions.

For preparation of suspension of antiviral agent and virus 1ml of 4HA viral suspension was added to 1ml of each of three concentrations (Lc, Mc and Hc) of every antiviral agent, in separate test tubes. Filtration was done by Millipore filter (pore size 0.22 µm) for each suspension separately.

Suspension of antiviral agent

To 1 ml of each of three concentrations (Lc, Mc, Hc) in case of every antiviral agent, 1 ml of normal saline solution was added in separate test tubes.

Evaluation of antiviral activity and mortality

For each concentration of antiviral agent 10 days old, 40 live embryonated broiler chicken eggs were divided into 6 groups named C1, C2, C3, D1, D2 and D3 with 5 eggs in each group. 0.2 ml of suspension of each concentration of antiviral agent (Lc, Mc, Hc) was introduced through allantoic route into each of 5 embryonated eggs of subgroups C1, C2 and C3 respectively in order to evaluate drug toxicity. On the other hand 0.2 ml of suspension of each concentration of antiviral agent (Lc, Mc, Hc) with virus was injected through allantoic route into each of 5 eggs of subgroups D1, D2, D3, respectively, in order to see antiviral efficacy. Groups A, comprising of 5 embryonated eggs will be served as negative control and each egg of this group will be treated with 0.2ml of normal saline solution. Similarly each of 5 eggs of group B (positive control) will be treated with 0.2 ml of viral suspention (1ml of 4HA unit+1ml of NS), under similar experimental conditions as in the case of groups C and D. After inoculation eggs were kept in incubator at 37°C with relative humidity of 60-70% for 48 h. After that eggs were chilled in refrigerator at 4°C for 12 h. Mortality of chick embryos was tested by candling the eggs of all groups and presence of virus was checked by rapid HA test.

RESULTS

Results showed that low concentration (50µg/ml) of amantadine was not lethal for the eggs in group C1 (Table I), but it did not stop the virus replication in all the eggs of group D1. Medium concentration (500 µg/ml) effectively stopped the virus growth in group D2, tested by haemagglutination test. All the 5 embryonated eggs in group C2 were alive showing that 500µg/ml concentration of amantadine was not toxic for chick embryos. High concentration of amantadine *i.e.* 1000 µg/ml along with stoppage of virus growth in group D3 also killed the embryos in both C3 and D3 groups, indicating that it was highly toxic for chick embryos. None of the 3 concentrations of opuntia were toxic for embryonated eggs in groups C1, C2 and C3. Moreover 4% and 8% extracts showed antiviral activity in groups D2 and D3, respectively.

It was noted that all the embryos were alive in these groups and allanto-amniotic fluid (AAF) showed no HA titre. Therefore, it was concluded that *Opuntia dellinii* has some antiviral compound in its composition, which is required to be investigated into. Results also indicated that neither of three concentrations (Lc, Mc and Hc) of green tea was toxic to the embryos of groups C1, C2 and C3. Furthermore only the high concentration (HC) *i.e.* 8g/100ml showed antiviral activity against AIV (type H7) in group D3, as all the eggs in this group were alive with no virus titre. Low (Lc) and medium (Mc) concentrations of green tea were not toxic for eggs, in groups C1 and C2 but did not stop the virus growth in groups D1 and D2, as the embryos in these groups were dead and AAF (allanto-amniotic fluid) caused agglutination of chick RBCs (5 % suspension), showing the presence of virus. All the three concentrations of papaya leaves extract were not toxic for the embryonated eggs in groups C1, C2 and C3, as all the embryos were alive. Secondly all the embryos in groups D1, D2 and D3 were dead and AAF showed positive haemagglutination activity, indicating the presence of virus. In case of positive (+) and negative (-) controls, all the embryonated eggs were live in group A (- control) with no haemagglutination activity. On the other hand all the embryos in group B (+ control) were dead with positive haemagglutination activity (Table II).

DISCUSSION

The present project was designed to evaluate antiviral efficacy of various antiviral agents including amantadine HCl, extracts of opuntia

Table II.- Comparative efficacy of various antiviral agents against avian influenza virus type H7N3/Pakistan/2003.

Antiviral agent	Conc. used µg/ml for Amantadine, g/100 for others	Antiviral agent + normal saline (Evaluation of toxicity)			Antiviral agent + 4HA virus (Evaluation of antiviral activity)		
		Sub-groups	Live/ total eggs	HA activity	Sub-groups	Live/ total eggs	HA activity
Amantadine HCl	50	C1	5/5	-	D1	0/5	-
	500	C2	5/5	-	D2	5/5	-
	1000	C3	0/5	-	D3	0/5	-
Extract of opuntia leaves	2	C1	5/5	-	D1	0/5	-
	4	C2	5/5	-	D2	5/5	-
	8	C3	5/5	-	D3	5/5	-
Extract of green tea	2	C1	5/5	-	D1	0/5	-
	4	C2	5/5	-	D2	0/5	-
	8	C3	5/5	-	D3	5/5	-
Extract of papaya leaves	2	C1	5/5	-	D1	0/5	-
	4	C2	5/5	-	D2	0/5	-
	8	C3	5/5	-	D3	0/5	-
Negative control[a]	**Group A** All the embryonated eggs in this group were alive with negative (-ve) HA activity.						
Positive control[b]	**Group B** All the embryonated eggs in this group were dead with positive (+ve) HA activity.						

A, normal saline; b, 4HA virus; HA activity, haemagglutination activity; +, positive haemagglutination activity; -, no haemagglutination activity. HA, 4HA titre of avian influenza virus (AIV) type H7N3/Pakistan/2003.

leaves, green tea leaves and papaya leaves, against avian influenza virus type H7N3/Pakistan/2003.

Of the anti influenza virus drugs available, the M2 inhibitors have a longer history of use than NA inhibitors and include amantadine and rimantadine. Amantadine's therapeutic antiviral spectrum is limited to influenza A virus. It's effectiveness is directly related to it's administration relative to the infection. The drug is 70-90% effective in preventing the infection if given at the time of exposure to the virus and can be used as a supplement to vaccination.

In this experiment three different concentrations of amantadine HCl *i.e*., 50, 500 and 1000 µg/ml were tested for their antiviral activity. A concentration of 1000 µg/ml was lethal for the chick embryos inspite of the fact that it successfully inhibited virus growth. The ideal concentration which strongly inhibited virus growth and did not kill the embryos was 500µg/ml.

Antiviral effect of amantadine against AIV was also reported by some other workers. Sherwood *et al*. (1999) reported that amantadine is effective against influenza. The only difference is the dose which might be due to the use of some different salt of amantadine by him. Staniova *et al*. (2001) also reported similar results that amantadine is an antiviral agent that specifically inhibits influenza A virus replication at a micromolar concentration. An analysis of reports of 52 randomized controlled trials by Jefferson *et al*. (2006), revealed that amantadine prevented 61% of influenza cases and 25% of cases of influenza like illness.

In this project a Pakistani variety of opuntia named *Opuntia dellinii* was also evaluated for its activity against avian influenza virus type H7N3/Pakistan/2003. Results revealed that it has excellent antiviral activity at concentrations of 4g and 8g/100ml. In addition to this 4g and 8g/ 100ml concentration were not toxic for chick embryos. A concentration of 2g/100ml observed as unable to block virus growth as well as non-toxic for embryos.

Similar results were observed by Ahmed *et al*. (1996) who used a Mexican variety named *Opuntia streptacantha* and declared that it is

antiviral against equine herpes, herpes simplex, influenza, respiratory syncytial virus and pseudorabies virus.

Green tea is a drink made from the steamed and dried leaves of the *Camellia sinensis* plant, a shrub native to Asia. Black tea is also made from this plant, but unlike green tea, it is made from leaves that have been fermented (which may reduce the level of some compounds such as antioxidants, in black tea). Green tea contains chemicals known as polyphenols, which have antioxidant properties. Catechins are the major group of polyphenols in green tea. The most important catechin seems to be EGCG. In this project three different concentrations of green tea extract were tested for their antiviral activity. Only the high concentration *i.e.* 8g/100ml was found to be effective in hindering influenza virus growth in embryonating broiler chicken eggs. Before this Imanishi *et al*. (2002) reported that green tea extract inhibits the growth of influenza virus by preventing it's adsorption. He further investigated that green tea extract exerts an additional inhibitory effect on acidification of intracellular compartments such as endosomes and lysosomes and thereby inhibits the growth of influenza A and B viruses in Madin –Darby canine kidney cells (MDCK). The effect was due to the (-) EGC, one of the major catechin molecules in green tea extract. Fassina *et al*. (2002) reported that increasing concentrations of EGCG, one of the components of green tea, strongly inhibited the replication of two different strains of virus. These studies are helpful in finding new herbs indigenous to Pakistan for prophylaxis and treatment of avian influenza.

Papaya is a tropical climate fruit. Typically, it is picked in a mature, green state, which is rich in two enzymes Papain and Chymonpapain (Zucker *et al.*, 1985). As a supplement to one's regular diet, the enzymes facilitate very powerful digestive action Regine *et al*. (2001) tested ethanol, water and acetone-diluted extracts of guava and papaya leaf sprouts on the bacteria in order to verify their microbicidal potential. The papaya leaf extracts *(Carica papaya*) showed no microbicidal activity. In this project three different concentrations of extract of fresh leaves of *Carica papaya* were tested for their antiviral efficacy. Neither of the concentrations was found to be effective against AIV type H7N3/Pakistan/2003. The concentrations used were 2, 4 and 8g/100ml. These concentrations were found to be safe for chick embryos.

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