Therapeutic Evaluation Of The Antiviral Activity Of *Azadirachta indica* In Comparison To Amantadine Hydrochloride Against Avian Influenza Virus Infection In Ducks

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ABSTRACT

Twelve AI isolates were isolated in ECE from diseased backyard ducks showed signs of depression, anorexia, greenish or watery diarrhea, severe neurological signs, and high mortalities. All isolates were identified using HIT to be H5. RT-PCR was used for detection and subtyping of AIVs using specific pairs of primers to amplify the hemagglutinin gene of subtype H5 and neuraminidase gene of subtype N1. Analysis of RNA extracted yielded positive reactions of correct size as H5 subtype specific primers (545 bp) and N1 subtype specific primers (616 bp). The efficacy of *Azadirachta indica* against AIV infection was evaluated in comparison to amantadine hydrochloride in 2-wk-old balady ducks inoculated with $0.1ml \times 10^6$ EID_{50}/ml of AIV H5N1. Aqueous Neem leaves extract provided ducks with 100% protection level, whereas neither clinical signs nor mortalities were recorded, while Adamine® presented 70% only.

INTRODUCTION

Avian influenza (AI), globally known as bird flu, became nowadays one of the most dangerous and alarming pandemic diseases allover the world. The Asian-lineage HPAI H5N1 produced fatal disease in poultry, wild birds, humans and other mammals, with subsequent spread to about 60 countries across three continents *(1)*. Despite the widespread in Eurasia, HPAI H5N1 was not present in Africa until 2006. In a matter of months, it spread quickly within the African countries. Unfortunately, Egypt, which reported its first HPAI H5N1 outbreak in 17 February 2006 *(2)*, had regular reporting of outbreaks in poultry in almost all of its governorates and the report of the General Organization for Veterinary Services (GOVS) in 7 July 2008 declared H5N1 to be endemic in Egypt *(3)*. The estimated loss of the Egyptian poultry industry after the first emergence of HPAI H5N1 in February 2006 was 2-3 billion US$. About 30 – 35 million birds were culled or depopulated and the income of 1.5 million people whose livelihoods depended on poultry was affected *(4)*.

All strains of influenza A viruses were perpetuated in the duck reservoir *(5, 6)*, which were usually nonpathogenic. The LPAI replicated in the respiratory tract and the intestine of such birds and lived in apparent harmony, causing no apparent disease signs *(7, 8)*. Many strains of HPAI H5N1 caused asymptomatic infection in ducks, making them a "Trojan horse" in the spread of those influenza viruses *(9, 10)*. However, in late 2002, outbreaks of HPAI H5N1 virus caused deaths of waterfowl *(10)* indicating that the biology of H5N1 influenza viruses in waterfowl had changed *(9)*.

Conventional intervention strategies against AI infection were based mainly on surveillance, stamping out of infected flocks, movement restriction, and enforcement of biosecurity measures *(11)*. Vaccination as a supportive tool in AIV control strategies was implemented to limit the spread of H5N1 and to reduce the losses *(12, 13, 14)*. However, in developed countries, and in consequence of their poor infrastructure, losses continued and the disease became endemic *(11)*. Recently,

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several approaches were developed to control the disease yielding promising results. Antiviral drugs were used to mitigate the catastrophic economical losses of AI outbreaks. Currently, two classes had been approved for the prevention and treatment of AI; the M2 channel inhibitors (amantadine and rimantadine) *(15; 16)* and the neuraminidase inhibitors (oseltamivir and zanamivir) *(17)*. However, prevalence of drug-resistant strains for both classes could undermine their clinical benefit *(18, 19,20)*.

Medicinal plants were part and parcel of human society to combat diseases, from the dawn of civilization. *Azadirachta indica*, commonly known as Neem, had attracted much attention within the worldwide medical community due to its wide range of medicinal properties. It was said to be the most promising tree of 21st century *(21)*. Neem was proved to be rich in flavonoids, triterpenoids and glycosides that were believed to be responsible for its antiviral potency *(22, 23, 24)*. Along with Neem's antiviral activity, it also had a highly effective immunostimulant effect *(21, 25, 26; 27)*.

Our study tried to gave an insight into an alternative approach for control of AI in poultry, with emphasis on HPAI H5N1 in ducks. The experimental study was designed to evaluate the therapeutic activity of *Azadirachta indica* (Neem) in comparison to amantadine hydrochloride (Adamine®) against AIV in ducks.

MATERIAL AND METHODS

Isolation of AIV

Samples collection

Brain, intestine, liver, spleen, pancreas, heart, lung, trachea, and kidney were collected aseptically from dead and sacrificed backyard ducks of different localities within Sharkia Province, Egypt during 2012. Ducks showed neurological signs, greenish or watery diarrhea, and high mortalities. Such ducks accompanied chicken flocks suffered previously from sudden onset of nervous signs with high mortality rates. Collected samples were stored at -20°c until used.

Virus isolation in ECE

Collected tissues were ground with a mortar and pestle in PBS with addition of antibiotic mixture (10,000 I.U. penicillin / ml + 10,000 µg streptomycin / ml+1000 µg gentamycin/ ml) to prepare a 10% tissue homogenate. Three times of repeated freezing and thawing were applied and then the homogenate was centrifuged at 3000 rpm for 20 min. Five, 9 - 11 days old, ECE were injected via allantoic sac with 0.2 ml of each clinical samples *(28)*.

Identification of AIV by HA and HIT

The harvested allantoic fluids (AF) were tested for their HA activity *(29)*. HA-positive AF were identified using standard antigen and antiserum against AIV of subtype H5 *(29)* which were kindly received from Dep. of Virolo., Zagazig University.

Identification and subtyping of AIV by RT-PCR

Positive HI samples were molecularly identified and subtyped by RT-PCR using specific pairs of primers to amplify the hemagglutinin gene of subtype H5 and neuraminidase gene of subtype N1. The H5 primers (H5-F 5´-**ACA CAT GCY CAR GAC ATA CT** -3´ and H5-R 5´-**CTY TGR TTY AGT GTT GAT GT** -3´) should amplify a **545** bp DNA *(30)*. The N1 primers (N1 F 5´-**TTG CTT GGT CGG CAA GTG C**-3´ and N1 R 5´-**CCA GTC CAC CCA TTT GGA TCC**-3´) should amplify a **616** bp DNA *(31)*.

Virus titration

The 50% egg infectious dose (EID50) was calculated *(32)*.

Histopathological examination

Brain, intestine, liver, spleen, heart, lung, trachea, and kidney were immediately fixed in 10% formalin solution, trimmed, washed and

dehydrated in ascending grades of alcohol, cleaned in xylene, embedded in paraffin, sectioned, and stained with Hematoxylen and Eosin stain (H&E) ***(33)***.

Therapeutic evaluation of antiviral activity of *Azadirachta indica* (Neem) in comparison to Adamine® against AIV infection in ducks

Agents

- Adamine®: 100 mg amantadine hydrochloride capsules, a product of Rameda™ Pharmaceutical Company, used as antiviral agent against Orthomyxoviuses at a dose of 25 mg/kg Bwt ***(34)***.
- Aqueous Neem leaves extract: Neem leaves were purchased from local markets in Sharkia province, Egypt. The 4% aqueous infusion was prepared ***(35)*** and administered *ad libidum* at a dose of 50 ml/liter ***(36)***.

Experimental design

Fifty, 1 day old balady ducklings were floor reared and fed on balanced rations to fulfill their nutritional requirement. None of the birds was vaccinated against AIV. At the age of 2 weeks, ducks were divided into 5 groups; 10 each, as follow;

Group (1): ducks were neither AIV infected nor treated (-ve control).

Group (2): 2-wk-old ducks were infected intranasally with $0.1ml \times 10^6$ EID_{50}/ml of isolated AIV H5N1 (+ve control).

Group (3): 2-wk-old ducks were infected intranasally with $0.1ml \times 10^6$ EID_{50}/ml of isolated AIV H5N1. After the onset of clinical signs, they received Adamine® at a dose of 25 mg/kg BWt orally *ad libidum* for 5 consecutive days.

Group (4): 2-wk-old ducks were infected intranasally with $0.1ml \times 10^6$ EID_{50}/ml of isolated AIV H5N1. After the onset of clinical signs, they received 4% aqueous Neem leaves extract at a dose of 50 ml/liter orally *ad libidum* for 5 consecutive days.

Group (5): 2-wk-old ducks were infected intranasally with $0.1ml \times 10^6$ EID_{50}/ml of isolated AIV H5N1. After the onset of clinical signs, they received 4% aqueous Neem leaves extract at a dose of 50 ml/liter and Adamine® at a dose of 25 mg/kg BWt orally *ad libidum* for 5 consecutive days.

Samples collection and preservation

Two blood samples from 5 ducks per group, 2.5 ml each, were collected via heart puncture. The 1st sample was collected in a test tube containing 50 I.U./ml blood heparin as anticoagulant. Collecting time was on the 14th (self-control), 21st, 28th, and 35th days of age to determine the lymphocytic output. The 2nd sample was collected in a centrifuge tube, left to clot, centrifuged at 2000 rpm for 10 m to allow serum separation, aspirated into cryovials, and stored at -20°C. Collecting time was on the 17th and 35th days of age for humoral immunity investigation and on the 14th (self-control), 21st, 28th, and 35th days of age for liver & kidney function tests.

Evaluation of *Azadirachta indica* efficacy

Clinical signs and lesions

From the 1st day of age and throughout the experimental period, clinical signs, mortality rate, and P.M. lesions were recorded daily for each group.

Growth performance

From the 1st day of age and throughout the experimental period, body weight (BWt), feed intake (FI) and feed conversion rate (FCR) were recorded weekly for each group.

Evaluation of immunological status

Cellular immunity

By estimation of lymphocytic output ***(37)***.

Humoral immunity

By colorometric estimations of serum IgM ***(38)*** and serum IgY ***(39)*** were carried out by spectrophotometer, using specific Vital® diagnostics inc. kits.

Evaluation of side effects

Effect on liver functions

By quantitative estimation of serum total proteins and albumen ***(40)*** and liver enzyme activities (ALT & AST) ***(41)***.

Effect on renal functions

By quantitative estimation of serum uric acid *(42)* and creatinine *(43)*.

Statistical Analysis

The obtained data were analyzed statistically using ANOVA test *(44)*.

RESULTS

Isolation of AIV

Twelve avian influenza isolates were isolated in ECE from diseased backyard ducks presented signs of depression, anorexia, greenish or watery diarrhea, severe neurological signs, and high mortalities. The most common gross lesions were congestion and hemorrhages of muscles and internal organs such as lungs, heart, liver, pancreas, proventriculus, duodenum, and brain. Necrotic foci in liver and pancreas with spleen atrophy and kidney nephrosis were also recorded. All of the inoculated samples caused embryos death with diffuse hemorrhages within 3-5 days post inoculation.

Identification of AIV by HA and HIT

The harvested allantoic fluids showed hemagglutination activity using slide HA against 10% wcRBCs and plate HA against 1% wcRBCs. The harvested allantoic fluids showed inhibition of agglutination of 1% wcRBCs using HIT with reference antigen and antiserum against AIV of subtype H5. All isolates were identified as AIV of subtype H5.

Identification and subtyping of AIV by RT-PCR

Analysis of extracted RNA yielded positive reactions of correct size as H5 subtype specific primers (545 bp) (Fig. 1a,b) and N1 subtype specific primers (616 bp) (Fig. 2a,b).

Fig. 1 a&b. PCR products of H5 subtype (545 bp in size) of amplified extracted AIV-RNA

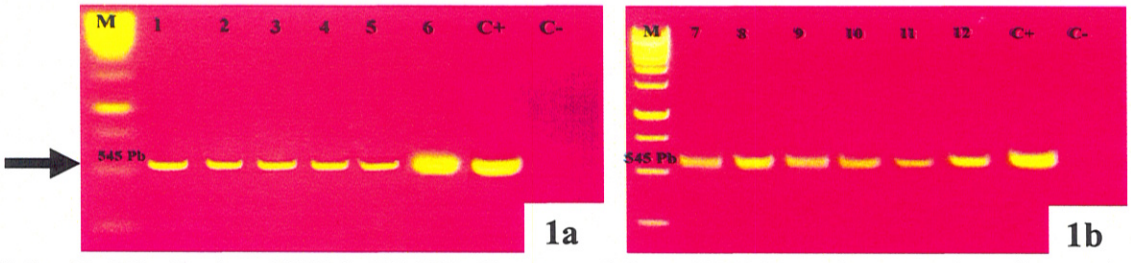

Fig1, a&b: H5 subtyping of AIVs by RT-PCR. The expected sizes of RT-PCR products were shown in parentheses. **Lane M;** ladder (100 bp), **lanes 1–12;** positive RT-PCR products **(545 bp), lane C+;** positive control, and **lane C-;** negative control.

Fig. 2 a&b. PCR products of N1 subtype (616 bp in size) of amplified extracted AIV-RNA

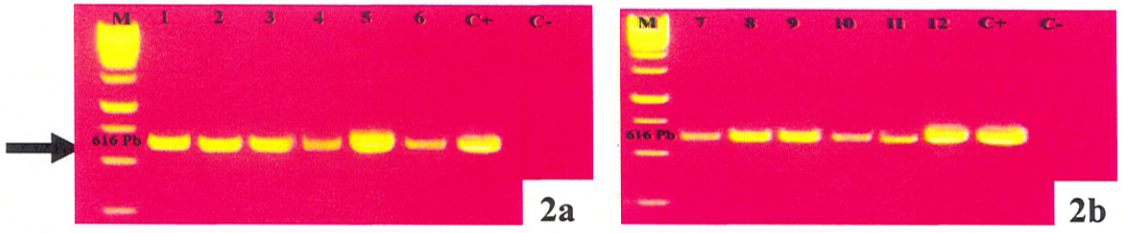

Fig. 2, a&b: N1 subtyping of AIVs by RT-PCR. The expected sizes of RT-PCR products were shown in parentheses. **Lane M;** ladder (100 bp), **lanes 1–12;** positive RT-PCR products **(616 bp), lane C+;** positive control, and **lane C-;** negative control.

Therapeutic evaluation of antiviral activity of *Azadirachta indica* (Neem) in comparison to Adamine® against AIV infection in ducks

Evaluation of *Azadirachta indica* efficacy

Clinical signs and lesions

Group (1) (-ve control)

Neither clinical signs nor mortalities were recorded. The ducks were active, eating and drinking normally, and gaining weight throughout the experimental period (Table 1).

Group (2) (+ve control)

Ducks showed signs of depression and anorexia as early as 3rd day post inoculation (DPI). The ducks were quiet and reluctant to move. All ducks suffered greenish diarrhea with mild to severe neurological signs beginning at 5th DPI characterized by tremors, uncontrollable shaking, marked loss of balance, lack of coordination, tilted head, seizures, and paralysis. Mortalities started on 7th DPI and the total mortalities were 6 ducks out of 10 (60%). Ducks that survived continued to have mild neurological signs until the end of the experiment. Those ducks ate and drank water, although they looked emaciated (Table 1). Grossly, the examined ducks showed picture of septicemia. The most common gross lesions were dehydration, petechial hemorrhages on serosal and abdominal fat and in body cavity. In addition, severe congestion of musculature, flaccid proventriculus, hemorrhagic enteritis, splenomegaly with necrotic foci, hepatomegaly with marbled appearance and necrotic foci, yellowish pancreas with petechial hemorrhages, dilated and flaccid heart with petechial hemorrhage on coronary fat, renomegaly with severe congestion, sometimes with urate deposits in tubules, severe hemorrhage and congestion of lungs, and congested brain were also recorded.

The predominant histopathological lesions of a 2-wk-old duck that died 7 days after intranasal inoculation with 0.1 ml of inoculum containing 10^6 EID_{50}/ml of isolated AIV H5N1 were found in the trachea, lung, intestine, liver, spleen, heart, brain, and kidney. The tracheal blood vessels were severely congested with degeneration and necrosis of the tracheal epithelium. Lung showed severe congestion of pulmonary blood vessels (Fig. 3a) in addition to massive hemorrhage with hyaline degeneration and lymphocytic infiltration (Fig. 3b). Intestinal blood vessels were severely congested with focal area of degeneration in the intestinal glands. The hepatic blood vessels and sinusoids were congested with perivascular lymphocytic infiltration (Fig. 3c, 3d). Hepatic tissue had coagulative necrosis with pyknotic nuclei (Fig. 3e). Splenic blood vessels were severely congested with depletion of lymphocytes from the white pulp and perivascular fibrosis and congestion. Myocardial blood vessels were severely congested. Cerebral blood vessels were severely congested (Fig. 3f) with hemorrhage and some degenerated neurons (Fig. 3g). Renal blood vessels showed severe congestion.

Group (3)

Ducks showed signs of depression anorexia as early as 3rd DPI. The ducks were quiet and reluctant to move. By the 4th DPI, the Adamine® treatment course began. Greenish diarrhea and neurological signs were noticed in 3 ducks by 5th DPI which died within three days after. The total mortalities were 3 ducks out of 10 (30%). Other ducks looked active, eating and drinking normally, and gaining weight throughout the experimental period (Table 1). The necropsy findings were similar to that reported in the 2nd group.

Group (4)

Ducks showed signs of depression and anorexia as early as 3rd DPI. The ducks were quiet and reluctant to move. By the 4th DPI, the aqueous Neem leaves extract treatment course began. Neither greenish diarrhea nor neurological signs were noticed. Ducks regained their activity by the end of treatment course (8th DPI). They ate and drank water normally, and continued to gain weight throughout the experimental period (Table 1).

Group (5)

Ducks showed signs of depression and anorexia as early as 3rd DPI. The ducks were quiet and reluctant to move. By the 4th DPI, the Adamine® and aqueous Neem leaves extract treatment course began. Neither greenish diarrhea nor neurological signs were noticed. Ducks regained their activity by the 3rd day of treatment course (6th DPI). They ate and drank water normally, and continued to gain weight throughout the experimental period (Table 1).

Growth performance

The growth performance results were summarized in Table 1.

Concerning body weight

- Ducks belonged to group (2) demonstrated significant decrease ($P<0.05$) in the mean values of body weight on the 21st, 28th, and 35th days of age compared with the –ve control group.
- Ducks in groups (3, 4, 5) showed significant increase ($P<0.05$) in the mean values of body weight on the 21st, 28th, and 35th days of age compared with group (2).
- Ducks belonged to group (4) demonstrated significant increase ($P<0.05$) in the mean values of body weight on the 21st, 28th, and 35th day of age compared with group (3).
- Ducks belonged to group (5) displayed significant increase ($P<0.05$) in the mean values of body weight on the 21st, 28th, and 35th days of age compared with groups (3, 4).

Concerning feed intake

- Ducks belonged to group (2) showed significant increase ($P<0.05$) in the mean values of feed intake on the 21st, 28th, and 35th days of age compared with the –ve control group.
- Ducks in groups (3, 4, 5) showed significant decrease ($P<0.05$) in the mean values of feed intake on the 21st, 28th, and 35th days of age compared with group (2).

Concerning feed conversion rate

- Ducks belonged to group (2) displayed significant increase ($P<0.05$) in the mean values of feed conversion rate on the 21st, 28th, and 35th days of age compared with the control –ve group.
- Ducks in groups (3, 4, 5) showed significant decrease ($P<0.05$) in the mean values of feed conversion rate on the 21st, 28th, and 35th days of age compared with the group (2).
- Ducks belonged to group (4) showed significant decrease ($P<0.05$) in the mean values of feed conversion rate on the 35th day of age compared with the group (3).
- Ducks belonged to group (5) displayed significant decrease ($P<0.05$) in the mean values of F.C.R. on the 21st, 28th, and 35th day of age compared with the groups (3, 4).

Evaluation of immunological status

The immunological response results were summarized in Table 2.

Concerning lymphocytic output

- Ducks belonged to group (2) demonstrated significant increase ($P<0.05$) in the mean values of lymphocytic output on the 21st and 28th day of age compared with the –ve control group.
- Ducks belonged to group (3) demonstrated significant decrease ($P<0.05$) in the mean values of lymphocytic output on the 21st and 28th day of age compared with the +ve control group. Ducks belonged to groups (4, 5) demonstrated significant increase ($P<0.05$) in the mean values of lymphocytic output on the 21st, 28th, and 35th day of age compared with the +ve control group.
- Ducks belonged to group (4) demonstrated significant increase ($P<0.05$) in the mean values of lymphocytic output on the 21st, 28th, and 35th day of age compared with the group (3).

- Ducks belonged to group (5) demonstrated significant increase ($P<0.05$) in the mean values of lymphocytic output on the 21st, 28th, and 35th day of age compared with the group (3). There were non significant changes ($P<0.05$) in the mean values of lymphocytic output in ducks belonged to group (5) and group (4) on the 21st, 28th, and 35th day of age.

Concerning humoral immunity

- Ducks belonged to group (2) demonstrated significant increase ($P<0.05$) in the mean values of IgM and IgY compared with the –ve control group.
- Ducks belonged to groups (3, 4, 5) demonstrated significant decrease ($P<0.05$) in the mean values of IgM and IgY compared with the +ve control group.
- Ducks belong to group (4) showed significant decrease ($p<0.05$) in the mean values of IgM and significant increase ($p<0.05$) in the mean values of IgY compared with group (3).
- Ducks belonged to group (5) demonstrated significant decrease ($p<0.05$) in the mean values of IgM compared with the groups (4, 3) respectively and significant increase ($p<0.05$) in the mean values of IgY compared with group (3). There were non significant changes ($P<0.05$) in the mean values of IgY in the ducks of groups (4, 5) compared with –ve control group.

Evaluation of side effects

Effect on liver functions

The liver function tests results were summarized in Table 3.

Concerning total proteins levels

- Ducks belonged to group (2) demonstrated significant increase ($P<0.05$) in the mean values of total proteins on the 21st day of age compared with the control –ve group.
- Ducks belonged to groups (3, 4, 5) demonstrated significant decrease ($P<0.05$) in the mean values of total proteins on the 21st day of age compared with the group (2).
- Ducks belonged to group (4) showed significant decrease ($P<0.05$) in the mean values of total proteins on the 21st day of age compared with group (3). There were non significant changes ($P<0.05$) in the mean values of total proteins in the ducks of groups (4, 5) on the 21st day of age compared with –ve control group.

Concerning albumin levels

- Ducks belonged to group (2) demonstrated significant decrease ($P<0.05$) in the mean values of albumin on the 21st day of age compared with the -ve control group.
- Ducks in groups (3, 4, 5) showed significant increase ($P<0.05$) in the mean values of albumin on the 21st day of age compared with group (2).
- Ducks belonged to group (4) showed significant increase ($P<0.05$) in the mean values of albumin on the 21st day of age compared with group (3). Non significant changes ($P<0.05$) were recorded in the mean values of albumin in the ducks of groups (4, 5) on the 21st day of age compared with –ve control group.

Concerning ALT & AST levels

- Ducks in group (2) showed significant increase ($P<0.05$) in the mean values of serum ALT & AST levels on the 21st, 28th, and 35th day of age compared with the -ve control group.
- Ducks in groups (3, 4, 5) showed significant decrease ($P<0.05$) in the mean values of serum ALT & AST levels on the 21st, 28th, and 35th day of age compared with group (2).
- Non significant changes ($P<0.05$) were recorded in the mean values of ALT in the ducks of groups (4, 5) on the 21st, 28th, and 35th day of age compared with –ve control group. Non significant changes ($P<0.05$) were recorded in the mean values of AST in the ducks of groups (4, 5) on the 21st

day of age compared with –ve control group.

Effect on kidney functions

The kidney function tests results were summarized in Table 4.

Concerning uric acid and creatinine levels

- Ducks belonged to group (2) demonstrated significant increase ($P<0.05$) in the mean values of uric acid and creatinine on the 21st, 28th, and 35th day of age compared with the –ve control group.
- Ducks belonged to groups (3, 4, 5) showed significant decrease ($P<0.05$) in the mean values of uric acid and creatinine on the 21st, 28th, and 35th day of age compared with the +ve control group.
- Ducks belonged to group (4) displayed significant decrease ($P<0.05$) in the mean values of uric acid on the 21st day of age compared with those in group (3).
- Non significant changes ($P<0.05$) were recorded in the mean values of uric acid and creatinine in the ducks of groups (4, 5) on the 21st, 28th, and 35th day of age compared with –ve control group.

Table 1. Effects of oral administration of aqueous Neem leaves infusion 4%; 50ml / liter and amantadine hydrochloride (Adamine®); 25 mg/kg B.W. of on growth performance in ducks experimentally infected with AIV of subtype H5N1

Age (D)	Group 1			Group 2			Group 3			Group 4			Group 5		
	B.W. (gm)	F.I. (gm)	F.C.R.	B.W. (gm)	F.I. (gm)	F.C.R.	B.W. (gm)	F.I. (gm)	F.C.R.	B.W. (gm)	F.I. (gm)	F.C.R.	B.W. (gm)	F.I. (gm)	F.C.R.
7	195± 5.88^{A}	180± 0.92^{A}	0.92± 0.16^{A}	175± 2.88^{A}	189± 1.25^{A}	1.08± 0.12^{A}	188± 3.17^{A}	185± 0.25^{A}	0.98± 0.06^{A}	179± 5.19^{A}	170± 2.60^{A}	0.94± 0.07^{A}	193± 10.96^{A}	174± 2.21^{A}	0.90± 0.05^{A}
14	523± 3.51^{A}	616± 1.350^{A}	1.17± 0.08^{A}	488± 4.32^{A}	605± 1.12^{A}	1.23± 0.13^{A}	467± 4.64^{A}	601± 0.98^{A}	1.28± 0.10^{A}	438± 4.57^{A}	590± 2.88^{A}	1.34± 0.13^{A}	438± 2.09^{A}	579± 3.50^{A}	1.32± 0.03^{A}
21	970± 7.00^{A}	980± 1.54^{E}	1.01± 0.01^{D}	615± 12.58^{E}	1160± 2.05^{A}	1.89± 0.03^{A}	695± 13.51^{D}	1000± 1.70^{D}	1.43± 0.07^{B}	750± 17.55^{C}	1050± 3.55^{C}	1.40± 0.03^{B}	890± 11.53^{B}	1110± 2.60^{B}	1.24± 0.01^{C}
28	1100± 2.45^{B}	1320± 2.15^{D}	1.20± 0.02^{C}	650± 15.02^{E}	1450± 3.25^{A}	2.20± 0.06^{A}	900± 5.17^{D}	1300± 1.53^{E}	1.44± 0.03^{C}	1020± 2.81^{C}	1350± 2.31^{B}	1.32± 0.04^{C}	1250± 3.95^{A}	1335± 2.18^{C}	1.07± 0.04^{D}
35	1330± 1.09^{C}	2320± 3.30^{C}	1.74± 0.001^{C}	800± 2.55^{E}	2860± 4.58^{A}	3.57± 0.07^{A}	1100± 3.65^{D}	2535± 3.25^{B}	2.30± 0.01^{B}	1590± 3.03^{B}	2150± 3.98^{D}	1.35± 0.004^{D}	1850± 6.38^{A}	2095± 1.48^{E}	1.13± 0.001^{E}

G (1) Non infected, non treated ducks (-ve control), **G (2)** AIV H5N1 infected ducks (+ve control), **G (3)** Infected ducks & treated with the Adamine®, **G (4)** Infected ducks & treated with the aqueous Neem leaves infusion 4%, **G (5)** Infected ducks & treated with by both Adamine® and aqueous Neem leaves infusion 4%.

Means carrying different superscripts in the same row were significant at $p< 0.05$.

Table 2. Effects of oral administration of aqueous Neem leaves infusion 4%; 50ml / liter and amantadine hydrochloride (Adamine®); 25 mg/kg B.W. of ducks on cellular immunity (L.O.) and humoral immunity (IgM & IgY) levels (mg/dl) in ducks experimentally infected with AIV of subtype H5N1

Groups	Lymphocytic output ($\times 10^5$/ml)				IgM (mg/dl)	IgY (mg/dl)
	Age in days				Age in days	
	14^{th}	21^{st}	28^{th}	35^{th}	17^{th}	35^{th}
1	1.76± 0.51^{A}	2.12± 0.29^{D}	1.84± 0.38^{D}	1.68± 0.36^{B}	7.67± 0.47^{E}	50.34± 2.86^{B}
2	1.97± 0.20^{A}	2.80± 0.30^{B}	2.54± 0.68^{B}	1.98± 0.23^{B}	34.38± 6.26^{A}	86.97± 11.16^{A}
3	1.90± 0.55^{A}	2.30± 0.50^{C}	2.46± 0.35^{C}	1.93± 0.80^{B}	16.07± 1.96^{B}	38.84± 1.74^{C}
4	1.8± 0.63^{A}	3.26± 0.31^{A}	3.67± 0.3^{A}	3.48± 0.61^{A}	13.16± 3.84^{C}	51.77± 2.79^{B}
5	2.33± 0.59^{A}	3.38± 0.52^{A}	3.83± 0.40^{A}	3.51± 0.70^{A}	10.87± 1.51^{D}	54.28± 6.35^{B}

G (1) Non infected, non treated ducks (-ve control), **G (2)** AIV H5N1 infected ducks (+ve control), **G (3)** Infected ducks & treated with the Adamine®, **G (4)** Infected ducks & treated with the aqueous Neem leaves infusion 4%, **G (5)** Infected ducks & treated with by both Adamine® and aqueous Neem leaves infusion 4%.
Means carrying different superscripts in the same column were significant at $p< 0.05$

Table 3. Effects of oral administration of aqueous Neem leaves infusion 4%; 50ml / liter and amantadine hydrochloride (Adamine®); 25 mg/kg B.W. of ducks on serum total proteins, albumin, ALT and AST levels (IU/L) in ducks experimentally infected with AIV of subtype H5N1

Groups	Total Protein (IU/L)				Albumin (IU/L)				ALT (IU/L)				AST (IU/L)			
	Age in days				Age in days				Age in days				Age in days			
	14^{th}	21^{st}	28^{th}	35^{th}	14^{th}	21^{st}	28^{th}	35^{th}	14^{th}	21^{st}	28^{th}	35^{th}	14^{th}	21^{st}	28^{th}	35^{th}
1	2.86± 0.35^{A}	2.65± 0.31^{B}	3.00± 0.13^{A}	2.80± 0.42^{B}	1.45± 0.02^{A}	1.38± 0.01^{A}	1.43± 0.06^{A}	1.44± 0.05^{A}	29.08± 2.08^{A}	29.3± 1.20^{C}	28.73± 0.46^{B}	29.14± 1.08^{B}	65.10± 4.84^{A}	68.34± 4.85^{C}	61.79± 3.46^{D}	61.69± 8.89^{C}
2	2.68± 0.27^{A}	4.93± 0.62^{A}	3.96± 0.23^{A}	4.03± 0.52^{A}	1.42± 0.09^{A}	0.98± 0.04^{C}	1.10± 0.17^{A}	1.34± 0.04^{A}	27.38± 2.52^{A}	65.3± 4.95^{A}	72.78± 7.59^{A}	69.04± 2.03^{A}	60.91± 7.54^{A}	94.47± 7.21^{A}	95.71± 3.45^{A}	103.1± 8.29^{A}
3	2.38± 0.80^{A}	3.46± 0.59^{C}	3.35± 0.64^{A}	3.34± 0.75^{AB}	1.40± 0.09^{A}	1.27± 0.06^{D}	1.36± 0.09^{A}	1.42± 0.08^{A}	27.59± 4.05^{A}	42.6± 1.29^{B}	35.49± 4.63^{B}	29.89± 0.88^{B}	62.72± 3.32^{A}	82.67± 5.74^{B}	80.84± 3.05^{B}	68.42± 1.87^{BC}
4	2.79± 0.31^{A}	2.59± 0.85^{B}	2.96± 0.77^{A}	3.05± 0.16^{B}	1.32± 0.04^{A}	1.38± 0.07^{A}	1.42± 0.03^{A}	1.47± 0.11^{A}	29.85± 3.59^{A}	35.7± 3.60^{BC}	36.66± 3.48^{B}	33.04± 2.46^{B}	65.93± 1.30^{A}	77.64± 8.34^{BC}	75.85± 3.96^{B}	73.42± 3.96^{B}
5	3.87± 0.30^{A}	2.98± 0.36^{B}	2.86± 0.34^{A}	2.78± 0.29^{B}	1.43± 0.01^{A}	1.45± 0.13^{A}	1.50± 0.38^{A}	1.56± 0.46^{A}	27.82± 2.17^{A}	34.7± 0.84^{BC}	32.52± 2.48^{B}	33.98± 1.46^{B}	60.24± 2.81^{A}	68.05± 5.37^{C}	69.70± 1.23^{C}	64.60± 3.70^{BC}

G (1) Non infected, non treated ducks (-ve control), **G (2)** AIV H5N1 infected ducks (+ve control), **G (3)** Infected ducks & treated with the Adamine®, **G (4)** Infected ducks & treated with the aqueous Neem leaves infusion 4%, **G (5)** Infected ducks & treated with by both Adamine® and aqueous Neem leaves infusion 4%.
Means carrying different superscripts in the same column were significant at $p< 0.05$

Fig.3. Histopathological lesions of duck tissues infected intranasally with 0.1 ml of inoculum containing 10^6 EID_{50}/ml of isolated AI H5N1

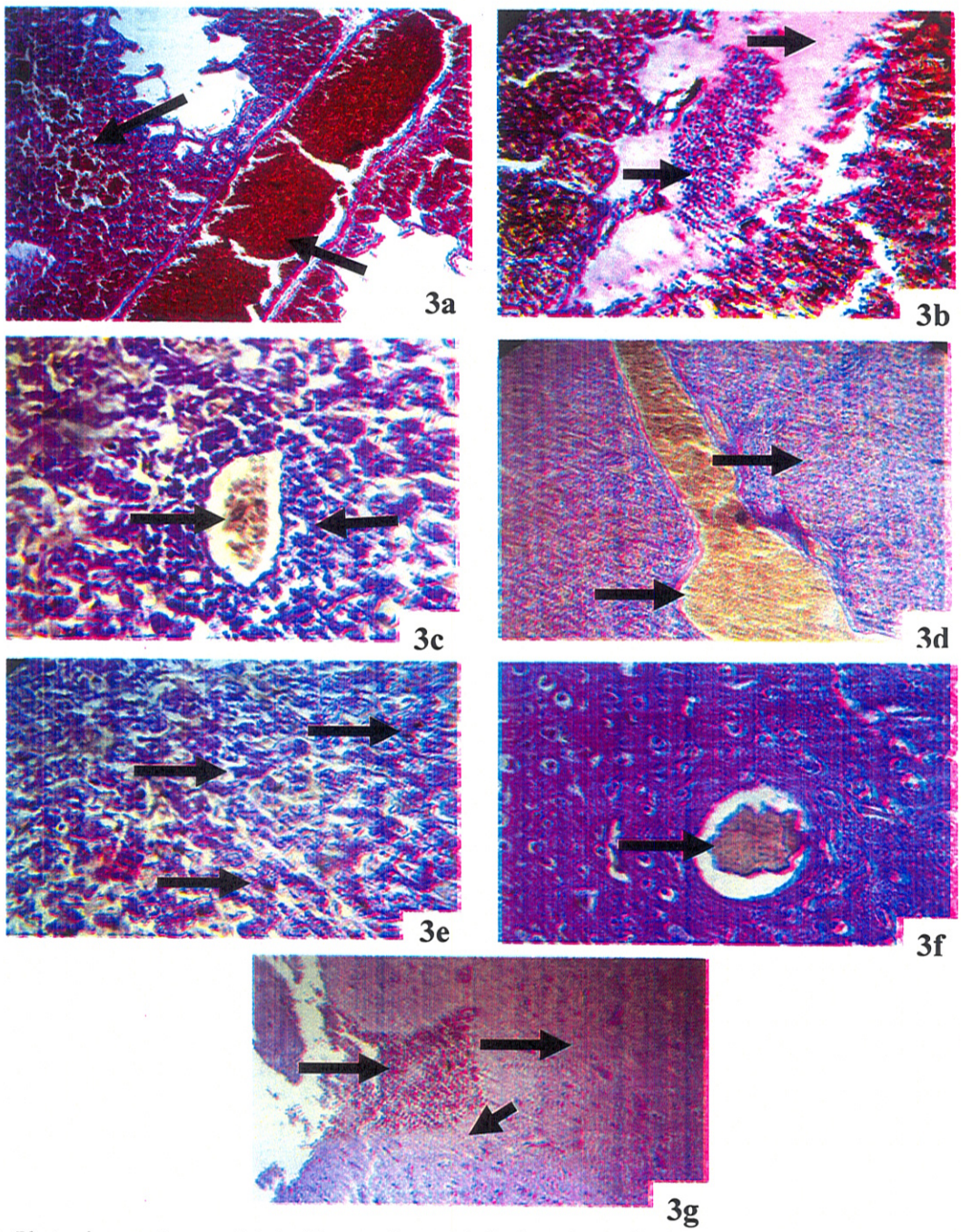

Fig. 3. Photomicrographs associated with experimental infection of a 2-wk-old Balady duck that died 7 days after inoculated with 0.1 ml of inoculum containing 10^6 EID_{50}/ml of isolated AIV H5N1. **3a;** severe congestion of pulmonary blood vessels (arrows). H&E., X 520. **3b;** massive hemorrhage with hyaline degeneration and lymphocytic infiltration of lung tissue (arrows). H&E., X 520. **3c;** congested hepatic blood vessels with perivascular lymphocytic infiltration (arrows). H&E., X 520. **3d;** congested hepatic blood vessels and sinusoids with focal lymphocytic cell infiltration (arrows). H&E., X 130. **3e;** coagulative necrosis in liver tissue with pyknotic nuclei (arrows). H&E., X 130. **3f;** severe congestion of cerebral blood vessels (arrow). H&E., X 520. **3g;** brain hemorrhage with some degenerated neurons (arrows). H&E., X 130.

Table 4. Effects of oral administration of aqueous Neem leaves infusion 4%; 50ml / liter and amantadine hydrochloride (Adamine®); 25 mg/kg B.W. of ducks on serum uric acid and creatinine levels (mg/dl) in ducks experimentally infected with AIV of subtype H5N1

Groups	Uric acid (mg/dl)				Creatinine (mg/dl)			
	Age in days				Age in days			
	14th	21st	28th	35th	14th	21st	28th	35th
1	6.60± 0.75^{A}	6.51± 0.97^{C}	7.03± 0.46^{B}	6.74± 0.61^{B}	0.39± 0.09^{A}	0.39± 0.03^{C}	0.40± 0.06^{CD}	0.51± 0.04^{BC}
2	6.74± 0.10^{A}	14.17± 1.13^{A}	12.86± 2.24^{A}	10.49± 1.15^{A}	0.45± 0.07^{A}	0.91± 0.04^{A}	0.88± 0.06^{A}	0.85± 0.10^{A}
3	6.82± 0.50^{A}	12.34± 1.48^{B}	9.28± 2.00^{B}	7.37± 0.51^{B}	0.42± 0.02^{A}	0.67± 0.10^{B}	0.62± 0.03^{B}	0.62± 0.07^{B}
4	7.04± 0.79^{A}	8.15± 1.03^{C}	7.72± 0.48^{B}	7.14± 0.24^{B}	0.34± 0.06^{A}	0.56± 0.06^{BC}	0.53± 0.10^{BC}	0.43± 0.08^{C}
5	6.83± 0.41^{A}	8.09± 0.88^{C}	7.29± 0.24^{B}	6.62± 0.29^{B}	0.47± 0.04^{A}	0.55± 0.09^{CD}	0.46± 0.05^{CD}	0.47± 0.03^{C}

G (1) Non infected, non treated ducks (-ve control), **G (2)** AIV H5N1 infected ducks (+ve control), **G (3)** Infected ducks & treated with the Adamine®, **G (4)** Infected ducks & treated with the aqueous Neem leaves infusion 4%, **G (5)** Infected ducks & treated with by both Adamine® and aqueous Neem leaves infusion 4%.

Means carrying different superscripts in the same column were significant at $p< 0.05$.

DISCUSSION

In our study, twelve AI isolates were isolated in ECE from diseased backyard ducks that presented suggestive signs of AIV infection in form of severe depression, anorexia, and greenish or watery diarrhea. Ducks displayed severe neurological signs with high mortality rates. Sinusitis, cloudy eyes, and blindness were also recorded. Necropsy findings revealed picture of septicemia. The most common gross lesions were congestion and hemorrhages of muscles and internal organs such as lungs, heart, liver, pancreas, proventriculus, duodenum, and brain. Necrotic foci in liver and pancreas with spleen atrophy and kidney nephrosis were also recorded. Those findings resembled that of other researchers who reported that ducks inoculated with HPAI H5N1 developed acute lethal disease, including severe depression, severe neurological dysfunction, and high mortality rates. The commonly recorded PM lesions were petechial hemorrhages in the duodenum, ceca, and proventriculus. Congestion of the duodenal mucosa with hemorrhagic content was observed. Hemorrhages of the pancreas and trachea were also present in some ducks *(10, 45, 46, 47, 48, 49)*. During the first record of HPAI H5N1 in Egypt, the clinical examination of ducks naturally infected showed severe nervous manifestations with mild signs of diarrhea and depression and sudden death. Sometimes there were no gross lesions because the bird died so quickly before development of predominant lesions. However, in most cases congestion and hemorrhages in almost all internal organs were recorded *(2, 50)*. Similar clinical signs and lesions continued to be reported in Egypt whether in experimentally infected *(51)* or naturally infected *(13, 52)* ducks.

Virus isolation remained the golden standard of virus diagnosis and characterization *(28, 53, 54)*. In this study, 12 AI isolates were successfully recovered from 12 tested flocks in 9 – 11d ECE via allantoic sac. Embryos died within 3-5 DPI showing diffuse hemorrhages indicating the presence of AIV *(28, 55)*.

The HA activity of the all harvested allantoic fluids was proved using slide and plate HA. Such results suggested that AIV was present in a high enough concentration to link together with erythrocytes forming a diffuse lattice *(29)*. The HA assay was not an identification assay, as other agents also had hemagglutinating properties. Other types of viruses, such as paramyxoviruses and adenovirus and certain bacteria also had hemagglutinating properties. The HA assay was followed by HIT to identify and determine the subtype of AIV *(56)*. All isolates were identified using HIT as AIV of subtype H5. The HI assay was a classical laboratory procedure for the classification or subtyping of hemagglutinating viruses. For AIV, the HI assay was used to identify the hemagglutinin (H) subtype of an unknown AI virus isolate or the HA subtype specificity of antibodies to AI virus *(29)*. In fact, the HI assay was a relatively inexpensive procedure utilizing standard laboratory equipment, less technical than molecular tests, and easily completed within several hours. However, when working with uncharacterized viruses or antibody subtypes, the library of reference reagents required for identifying antigentically distinct AI viruses and/or antibody specificities from multiple lineages of a single hemagglutinin subtype required extensive laboratory support for the production and optimization of reagents *(57)*.

A one-step RT-PCR was used for detection and subtyping of AIVs using specific pairs of primers to amplify the H5 and N1 genes. Analysis of extracted RNA yielded positive reactions of correct size as H5 specific primers (545 bp) *(30)* and N1 specific primers (616 bp) *(31)*. RT-PCR in the last few years, was established as the most accurate and reliable assay for detection of H5N1 in clinical samples. It combined the speed and simplicity of rapid antigen detection assays with the higher sensitivity and specificity of virus isolation *(30; 58)*. It also allowed detection of viral RNA below cultivable levels of viruses and in specimens in which the virus might not be viable *(59)*. Another major advantage of RT-PCR was the rapidity with which typing and subtyping of HA in a single RT-PCR step could be performed and, if desired, simultaneous subtyping of NA in the same cDNA template *(31)*. The use of PCR for detection of AIV had the advantage over egg inoculation method of being easier and faster. Propagation of AIV in ECEs required much time as embryos had to be incubated 9 – 11 days prior to use *(60)*. In addition, the persistent propagation in ECEs was incriminated in the emergence of mutations viruses *(61)*. The RT-PCR was as sensitive as ECE inoculation followed by serological identification *(62)* however, due to frequent mutations in influenza viruses; primer sequences had to be appropriate for currently circulating strains *(63)*.

Taking into consideration that the pathogenicity of HPAI H5N1 in domestic ducks was affected by the age of the ducks, with younger ducks showin a more severe disease *(47,48, 64)*, and route of inoculation, with more lethality in case of intranasal as compared to oral route *(65)*, a pathogenicity study in 2-wk-old balady ducks was performed following standard protocol of OIE, 2008 *(29)*. Ducks were divided into 5 groups, 10 each; a control group (negative control) and four groups inoculated intranasally with $0.1ml \times 10^6$ EID_{50}/ml of AIV that was isolated and identified during our study to be of subtype H5N1. One group was let untreated (positive control), while others received Adamine®, aqueous Neem leaves extract, and a mixture of them respectively. Neither clinical signs nor mortalities were observed in the negative control ducks (Group 1). Control positive ducks (Group 2) showed signs of depression and anorexia 3 DPI. Greenish diarrhea with mild to severe neurological signs were recorded beginning at 5^{th} DPI. Mortalities started on 7^{th} DPI that reached 60%. Ducks that survived continued to have mild neurological signs and looked emaciated until the end of the

experiment. Grossly, the examined ducks showed picture of septicemia. The most common gross lesions were dehydration, petechial hemorrhages on serosal and abdominal fat and in body cavity, severe congestion, and hemorrhages of musculature and internal organs such as intestine, liver, spleen, pancreas, heart, kidney, lungs, and brain. Liver and spleen showed necrotic foci. Ducks belonged to groups 3, 4, and 5 began their treatment course just after the onset of the clinical signs (i.e. 4th DPI). Clinical signs proceeded in ducks of group 3 in form of greenish diarrhea and neurological signs with 30 % mortality rate, which were not recorded in group 4 and 5.

Those results agreed with that of other researchers who mentioned that ducks infected with HPAI H5N1 developed acute lethal disease, including severe depression, severe neurological dysfunction, and high mortality rates with septicemic PM pictures ***(51, 65, 66)***. The virulence of HPAIVs H5N1 affected the severity of clinical signs and mortality rates. Isolated HPAI H5N1 from Egypt in 2007 (A/chicken/Egypt/9402 /2007) caused 37.5 % mortalities in Pekin ducks, while the 2008 isolate (A/chicken/Egypt/08124S/2008) caused 100% mortalities ***(51)***. Pantin-Jackwood et al., (2012) declared that mortality rates swung around 87.5% (A/Vietnam/1203/04), 100% (A/Crow/Thailand/04), 87.5 % (A/Egret/HK/757.2/02), and 37.5% (A/Thailand PB/6231/04) in Pekin ducks inoculated with different viruses of subtype H5N1***(48)***.

In this study, the predominant histopathological lesions of a 2-wk-old duck that died 7 days after inoculated intranasally with 0.1 ml$\times 10^6$ EID_{50}/ml of isolated AIV H5N1 were present in trachea, lung, intestine, liver, spleen, heart, brain, and kidney as describe in Figure 3a to 3g. Those findings agreed with the previous statement of other researchers***(47, 50)*** . In the brain, randomly scattered foci of malacia with gliosis lymphoplasmacytic perivascular cuffs were present. Perivascular edema and hemorrhage were observed. Neuronal degeneration and necrosis were also present. In the heart, myocardial degeneration to necrosis was observed. The trachea showed degenerative epithelium and mild lymphocytic infiltration in the submucosa. The lung had consisting of congestion and interstitial inflammation with mixed mononuclear cells. The intestinal epithelium showed inflammatory changes in the lamina propria. Necrosis of scattered hepatocytes with sinusoidal histiocytosis and congested blood vessels were observed in the liver. The spleen had lymphoid depletion ranging from apoptosis to necrosis in remaining lymphocytes ***(47, 50)***. Eventually, it should be clear that specific histopathologic descriptions for experimental studies varied with individual viruses because of variations in inoculum doses, strain of the bird, route of inoculation, and passage history ***(67)***.

The protection level of amantadine hydrochloride (Adamine®) in ducks experimentally infected intranasally with 0.1ml$\times 10^6$ EID_{50}/ml of isolated AI H5N1 (Group 3) was 70%. Greenish diarrhea and neurological signs were noticed in three out of ten ducks, which died at the end. Aqueous Neem leaves extract provided ducks of group 4 and 5 with 100 % protection level, whereas neither clinical signs nor mortalities were recorded.

Several researchers studied the efficacy of prophylactic and/or therapeutic administration of amantadine hydrochloride in poultry against HPAIV and their findings fit ours. Although efficacy rates of 70 - 90 % for amantadine against influenza type A in poultry had been reported ***(68, 69, 70)***, several drawbacks might hinder its extensive application worldwide: (1) Optimum prophylaxis was obtained only when amantadine was administered in an adequate, uninterrupted, and sustained amount from at least 2 to 23 DPI. Amantadine given in drinking water in such manner was efficacious to decrease morbidity, mortality, transmissibility and limit decrease in egg production ***(71)***. (2) Nonetheless, recovered birds were susceptible to reinfection and subclinical infection was reported in most of treated birds ***(69,72)***. (3)

Importantly, amantadine lost its effectiveness as amantadine-resistant mutants emerged within 2 – 3 days of treatment and killed all in-contact chickens. Amantadine resistant strains were irreversible, stable, and transmissible with pathogenic potential comparable to the wild-type virus. Even more, the resistant mutants replaced the wild-type virus and became dominant *(72)*. (4) Notably, The recent prevalence of amantadine resistance among A/H3N2 and A/H1N1 viruses circulating worldwide *(19)*, as well as among A/H5N1 viruses *(73)*, meant hazards of kicking out cornerstone an antiviral in case of pandemic *(74)*. Therefore, the extra-label use of amantadine in poultry was banned by all concerned international organizations *(75)*.

Neem was proved to be rich in flavonoids, triterpenoids and glycosides that were important constituents of many traditional herbal medicines. Such bioactive compound of Neem were believed to be responsible for the herb's direct virucidal as confirmed by the absence of any virus-related symptoms *(22, 23, 24)*: Its aqueous leaf extract showed antiviral activity against Newcastle Diseases Virus (NDV) *(21)*, and Infectious Bursal Disease (IBD) *(27)*. Several flavonoids had been reported to have anti-influenza virus effect by inhibiting Neuraminidase surface antigens activities responsible for catalyzing the cleavage of neuraminic acid residues to facilitate the movement of the virus to and from sites of infection in the respiratory tract *(76)*. Synergism had also been reported between flavonoids and other antiviral agents giving a more active agent than its individual component *(77)* and that might explain the superior results obtained in ducks of group (5) in comparison to those of group (4) and group (3).

In poultry, many herbs species with potential antiviral and immunoadjuvant effects against AIVs were frequently described using *in-vitro* cell culture methods and ECE or *in-vivo* mouse models *(78, 79)* giving similar efficacy levels that obtained in our study. *Eugenia jambolana* extracts, for instance, had 100% virucidal activity against HPAIV H5N1 in tissue culture and in ECE *(80)*. Likewise, eucalyptus and peppermint essential oils preparations protected broilers against H9N2 *(81)*. Moreover, application of lyophilized green tea by-product extracts namely catechins in reduced H9N2 virus replication and excretion in experimentally infected chickens *(82)*.

Concerning the effect of aqueous Neem leaves extract on growth performance; our results revealed that ducks belonged to groups (5, 4) showed better results compared with those of group (3) or control groups respectively. Those results coincided with those of Chakravarty and Prasad (1991) who achieved highest body weight gain and best feed conversion ratio as compared to control when offered Neem leaf extract to broilers *(83)*. The increase in weight gain could be possibly due to the presence of macro minerals such as Potassium, Magnesium, and Phosphorous in addition to micro minerals such as Iron, Copper, Manganese, and Zinc in the extract *(84)*. The deficiency of those minerals resulted in anorexia, osteoporosis, and retarded growth *(85)*. Antimicrobial activity of Neem might substitute the existing antibiotic growth promoters *(86)*. Aqueous Neem leaves extract was proved to suppress many pathogenic bacteria including *Staphyloccoccus aureus, Mycobacteria spp, Salmonella paratyphi, and Klebsiella pneumoniae (23)* in addition to many fungal species including *Epidermophyton, Trichophyton, Microsporum, and candida* (*87)* and thus resulted in enhanced growth. Its diversified effect on intestinal micro flora could be another reason *(36)*.

Regarding the effect of aqueous Neem leaves extract on the immune response, the results showed that, ducks belonged to groups (5, 4) showed significant increase ($p<0.05$) in the mean values of lymphocytic output compared with those belonged to groups (3) respectively. Ducks belonged to group (5) demonstrated significant decrease ($p<0.05$) in the mean values of IgM compared with the groups (4, 3) respectively and significant increase ($p<0.05$) in the mean values of IgY compared with group (3). Aqueous Neem extract activated both the alternative as well as the classical pathway of complement system

(88). In addition, neem extract was proved to cause an elevation of both immunoreactive and bioactive TNF-alpha and gamma-interferon in serum. Whereas neem extract boosted the lymphocytic immune system, it antagonized many disease-causing organisms such as bacteria, viruses, and fungi resulting in significantly higher humoral immune responses *(89)*. Neem oil had been shown to selectively activate the cell-mediated immune mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge *(90, 91)*. Our findings fit that of previous researchers who proved that the aqueous extract of leaf possessed potent immunostimulant activity as evidenced by both humoral and cell-mediated responses. Leaf extract at 100 mg/kg after three weeks of oral administration causes higher IgM and IgG levels. Neem significantly enhanced antibodies titers against the Newcastle Disease Virus (NDV) after challenging with Infectious Bursal Disease Virus (IBDV) and feeding the powdered dry leaves of Neem at a rate of 2 g/kg of ration *(92)* or supplying 4% Neem leaves infusion at level of 50ml L^{-1} of fresh drinking water *(36)*. Aqueous extract of Neem leaves provided higher antibody titers against Newcastle Diseases (ND), Infectious Bronchitis (IB), and Infectious Bursal Disease (IBD) *(26)*. Broiler chicks fed Neem fruits at rate of 7 g/kg of ration showed higher antibody titers against Newcastle Diseases (ND), and Avian Influenza (AI) *(25)*.

Regarding the effect of aqueous Neem leaves extract on liver and renal functions, ducks belonged to groups (3, 4, 5) demonstrated significant decrease ($P<0.05$) in the mean values of total proteins, serum ALT, and AST. Ducks also showed significant increase ($P<0.05$) in the mean values of albumin compared with the +ve control group. Ducks belonged to groups (3, 4, 5) demonstrated significant decrease ($P<0.05$) in the mean values of uric acid, and creatinine compared with the +ve control group. Generally, serum levels of ALT and AST had been reported to be sensitive indicators of liver injury. The disturbance in the transport function of the hepatocytes as a result of hepatic injury caused the leakage of enzymes from cells due to altered permeability of membrane resulting in decreased levels of ALT and AST in the hepatic cells and a raised level in serum. In addition, elevated serum levels of uric acid and creatinine could be considered as indicators of kidney damage *(93)*. Our study proved that administration of aqueous Neem leaves extract significantly reduced serum levels of ALT, AST, uric acid, and creatinine representing an appreciated hepatorenal protection level. Our findings fell in line with that of other researchers who admitted the hepatoprotective activity of aqueous extracts of Neem leaves lowering the seum levels of ALT and AST *(94, 95, 96)* in addition to serum levels of urea and creatinine *(97, 98)*. The hepatorenal protection ability of Neem extract could be elucidated by stabilizing serum level of marker enzymes responsible for such damage *(99)*. It might also be attributed to other biological activities of the plant extract, such as antioxidant activity *(86)*. The process, through which free radicals were liberated, could be considered as a normal function of the body, but the resulting molecules were unstable and could damage any other cells. A series of disorders, including liver and kidney diseases, occurred due to high level of free radicals. Since it had been shown to be rich in flavonoids, which were well known antioxidants, neem protected against such damage by boosting antioxidant levels *(100)*.

From the obtained results, it was concluded that:

- AIV present in Egypt was of subtype H5N1, which was highly pathogenic in ducks whereas it caused appearance of the typical sings of HPAIV H5N1 with high mortality post experimental inoculation of ducks.
- In addition to its antiviral and immunostimulant activity, aqueous Neem leaves extract often had antibacterial, antifungal, antioxidant, hepatoprotective, and renoprotective properties, which might provide alternative natural broad-spectrum therapy for control of AIV in poultry farms.

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الملخص العربي

التقييم العلاجي لمستخلص نبات النيم المائي كمضاد لعدوى فيروس انفلونزا الطيور في البط مقارنة بالأمانتادين هيدروكلوريد (أدامين)

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في هذه الدراسة تم عزل اثنتا عشرة عترة لفيروس انفلونزا الطيور بالحقن في أجنة البيض المخصب عمر ٩ – ١١ يوم من قطعان بط منزلية، كانت قد ظهرت عليه بعض الأعراض العصبية و إسهال أخضر او مائي مع نسب نفوق عاليه. و بواسطة اختبار منع التلازن الدموي تم تصنيف المعزولات الفيروسية وكانت من نوع انفلونزا الطيور اتش ٥. كما تم استخدام تفاعل البلمرة المتسلسل لتاكيد التصنيف وذلك باستخدام بادىء مخصص لكل من الجين اتش ٥ والجين إن ١ وقد أعطى الاختبار نتائج ايجابية للحجم المخصص للجين اتش ٥ و الجين إن ١ مما أكد على أن المعزولات الفيروسية كانت لفيروس انفلونزا الطيور من النوع اتش ٥ إن ١. تم تقييم كفاءة المستخلص المائي لنبات النيم كمضاد لفيروس انفلونزا الطيور في البط وذلك من خلال عمل عدوى صناعية في بط بلدي عمر أسبوعان بجرعة ١, مللى ×١٠ ^{٦} من فيروس الأنفلونزا اتش ٥ إن ١ عن طريق الانف . كانت نسبة الحماية باستخدام المستخلص المائي لنبات النيم هي ١٠٠% حيث لم تظهر أي اعراض او نفوق في البط المعدي بينما كانت النسبة ٧٠% فقط في حالة الأدامين.