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RESEARCH ARTICLE

Evaluation of Antiviral Activity of *Azadirachta indica* (Neem) Bark Extract against Newcastle Disease Virus

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ABSTRACT

In the present research, the *in-vitro* and *in-ovo* antiviral activity of Neem (*Azadirachta indica*) bark extract against Newcastle disease virus (NDV) was evaluated. Various dilutions of *Azadirachta indica* extract were used against NDV. *In-vitro* evaluation was done by performing spot assay and micro-hemagglutination test, while *in-ovo* antiviral activity was assessed by injecting the extracts in 11 days old embryonated eggs. During *in-vitro* evaluation, it was found that the stock solution and 1:2 dilution of Neem bark extract exhibit antiviral activity but at the same time these concentrations showed cytotoxic activity as well, while higher dilution (1:8) showed non-significant antiviral activity. Same was true for *in-ovo* evaluation, higher dilutions showed non-significant activity. Taking together, it may be concluded there was no significant difference in the antiviral effects of different concentrations of Neem bark extracts but the exposure time was a significant variable for cytotoxicity.

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INTRODUCTION

Poultry is the second largest industry of Pakistan but its progress was hampered due to many bacterial and viral diseases which are major constraints for profitable poultry production (Ahmed *et al.*, 2009). Among viral diseases, the most significant is Newcastle disease (ND). Newcastle disease virus (NDV) is designated APMV-1.

NDV is grouped into five pathotypes (Alexander and Senne, 2008; Alexander, 2010), which include Viscerotropic, Neurotropic, Velogenic, Mesogenic and Lentogenic. Since its acknowledgment in 1926, ND is counted as being endemic in many countries. Regardless of vaccination, other prevention and control measures are necessary to prevent ND outbreaks. Every year, ND causes destruction of poultry industry in Pakistan, hence affects the economy of country badly. Due to aerosol transmission and the resistance of the virus in the environment, NDV causes infection in other birds (Rasool *et al.*, 2015; Pansota *et al*., 2013; Sattar *et al.*, 2016).

General toxicity of various antimicrobial agents is a serious concern that is why alternate antiviral therapies are now the area of interest for the virologists. The natural plant products exhibit an imperative role in disease management and control (Mahmood *et al*., 2016). Different effects posed by these products include microbial growth inhibition, antioxidant activity and genetic/molecular pathways modulation. Neem (*Azadirachta indica*), is a member of the family Meliaceae and commonly found in Pakistan, India, Nepal and Bangladesh. Various phyto-ingredietns of *Azadirachta indica* including limonoids, nimbin and nimbolide, and due to these constituents Neem plays a satisfactory role in treatment of infectious diseases (Tiwari *et al*., 2010). Aniseed has been reported to be immunostimulant against Newcastle disease virus (Mahmood *et al*., 2014 a & b). Quercetin and ß-sitosterol were reported as first purified polyphenolic flavonoids from fresh Neem leaves and were recognized as antimicrobial agents. Various spices and their oils have been used to treat several microbial infections (Naveed *et al*., 2013 a & b) and green tea extract also posses antiviral effects (Aslam *et al*., 2014). Various studies about the antiviral potential of Chinese medicinal herbs against many other viruses including NDV have also been reported (Li *et al.*, 2005).

MATERIALS AND METHODS

Plant material: The fresh bark was collected from Neem tree. The help of a botanist at the Department of Botany

University of Agriculture Faisalabad was seeked for identification. The bark was shade dried and grounded into a coarse powder using a grinder (0.25 mm sieve).

The stock of Neem bark powder was dissolved in PBS in a 50 ml tube and kept on a rotatory shaker at 4°C overnight. Red-brown supernatant was obtained after centrifugation at 3000 rpm (10 min), filtered through 0.22µm membrane filter and stored at 4°C until used further (Tiwari *et al.*, 2010).

Chicken Red Blood Cells (RBCs): The chicken blood was taken in sterilized vacutainers (with anti-coagulant). The RBCs were washed, and 1% RBCs suspension was prepared following Rasool *et al.*, (2015). These RBCs suspensions were used in Spot and Plate hemagglutination tests, respectively.

Preparation of inoculum (2-fold dilution of virus and Neem bark extract): A 0.5 ml of 4HAU of locally isolated and characterized NDV isolated from field outbreak and maintained at Institute of Microbiology was mixed separately with 0.5 ml of stock solution, 1:2, 1:4 and 1:8 dilutions of Neem bark extract in sterile Eppendorf tubes and incubated for 60 minutes at 37°C. After incubation, 0.2 ml of each suspension was inoculated into a group of ten eggs and incubated for 3 days. Candling was done daily, and observations were recorded. Allantoic fluid was harvested on 4th day for hemagglutination test (Mabiki *et al.*, 2013).

***In-vitro* antiviral activity:** *In-vitro* antiviral activity was checked by incubating the NDV with different concentrations of Neem bark extract in Eppendorf tubes. Spot hemagglutination test with 25% RBCs suspension was conducted post-incubation along with negative control (NDV only) as described by Chen *et al.* (2013).

Spot assay: Spot assay was performed by using 25% chicken RBCs on glass slide (Beck *et al*., 2009). A 50 µl of 25% chicken RBCs were mixed with 50 µl of pre-incubated NDV having different concentrations i.e., 1:2, 1:4 and 1:8 of Neem bark extract and observed for agglutination. Absence of agglutination of RBCs by virus was related with the antiviral activity of Neem bark extract.

Micro-hemagglutination test: Micro-hemagglutination test was performed by using the 1% RBCs in 96 well microtitration plate. A 50 µl of pre-incubated suspension of 4HAU of local Velogenic NDV and different concentrations of Neem bark extract were poured into the wells of microtitration plate and then 50 µl of 1% RBCs were poured in all wells containing the suspensions. The 1st well was used as virus control and 12th well as RBCs control. Inhibition of hemagglutination activity of NDV (button formation) was related with antiviral activity of Neem bark extract (Siddique *et al*., 2015).

***In-ovo* antiviral and cytotoxicity activity:** Vero cells were propagated in minimal essential medium (MEM, Sigma, USA) supplemented with 10% fetal bovine serum (FBS). ND virus was grown in vero cells and cell free virus supernatant was separated and stored at -70°C for further use.

Maximum non-toxic concentration (MNTC) of Neem extract and control (Amantadine, Sigma, USA) was estimated on the basis of alteration in cellular morphology (Swayne and King, 2003). The highest dilution of extract without any notifiable structural change in the cells up to 96 hours was considered as MNTC. The embryonated chicken eggs (ECEs) were inoculated with MNTC, via allantoic route along with positive and negative control and incubated at 37°C with 70% humidity for 96 hours. Mean HA titer of infected eggs was calculated (Sood *et al*., 2012; Ong *et al*., 2014).

In a separate experiment, *In-ovo* antiviral activity was evaluated in 9 to11 day-old ECEs. For this purpose, two types of inoculums were prepared. The first inoculum was prepared by mixing 0.5 ml of virus with 0.5 ml of different concentrations i.e., 1:2, 1:4 and 1:8 of Neem bark extract.

The antiviral activity was determined through micro-hemagglutination test following Lanre *et al.* (2011) and Mabiki *et al.* (2013).

$$\% \text{ MEW} = \frac{\text{MEW of the harvested embryo from treated ECE}}{\text{MEW of the harvested embryo from untreated ECE}} \times 100$$

Where, MEW is Mean Embryo Weight.

RESULTS

Spot hemagglutination test: In spot assay, stock solution of Neem bark extract and 1:2 dilutions inhibited the visible clumping/agglutination of chicken RBCs, which shows the antiviral activity. However, the higher dilution of 1:8 did not show antiviral activity (Table 1).

The stock solution, 1:2 and 1:4 dilution of Neem bark extract showed antiviral activity and blood discoloration has also been observed. The higher dilutions of Neem bark extract (1:8) neither showed agglutination of RBCs by NDV nor the change in color.

Table 1: *In-ovo* antiviral activity of Neem bark extract

Extract	Total number of eggs infected	Mean HA titer in infected eggs (log_2)	Reduction in HA titer in infected eggs % (log_2)
Untreated virus control	5	8.0	--
Neem bark extract treated	5	5.0	88%

Table 2: Hemagglutination titers of NDV Pre-inoculation and harvested allantoic fluids post-inoculation with NDV and different concentrations of Neem bark extract

Group	Treatment	HA titer of Velogenic NDV used *In-ovo*	HA titer of NDV in harvested allantoic fluid	Reduced HA (%)= $\frac{*C-*E}{*C} \times 100$
A	Stock solution of Neem bark extract	1:64	0	100%
B	1:2 dilution	1:64	1:32	93.75%
C	1:4 dilution	1:64	1:128	75%
D	1:8 dilution	1:64	1:512	0%
E	Positive control for virus	1:64	1:512	---
F	Positive control for Neem bark extract	----	----	---
G	Negative control	----	----	---

*C=Hemagglutination titer of virus control group; *E=Hemagglutination titer of treated embryonated chicken eggs group.

Table 3: Percent mean embryo weight in embryonated chicken eggs post-inoculation with NDV and different concentrations of Neem bark extract

Groups	Treatment	Total No. of ECE in group (n)	Total weight of ECE in grams	Mean embryo weight = $\frac{Total\ weight}{n}$ ± S.E
A	Stock solution of Neem bark extract	10	397.45	$\frac{397.45}{10} = 39.74^{bc} \pm 0.49$
B	1:2 dilution	10	385.75	$\frac{385.75}{10} = 38.575^{cd} \pm 0.47$
C	1:4 dilution	10	380.80	$\frac{380.80}{10} = 38.08^{de} \pm 0.43$
D	1:8 dilution	10	377.20	$\frac{377.20}{10} = 37.72^{de} \pm 0.40$
E	Positive control for virus	10	371.85	$\frac{371.85}{10} = 37.185^{e} \pm 0.34$
F	Positive control for Neem bark extract	10	372.89	$\frac{372.89}{10} = 37.28^{e} \pm 0.40$
G	Negative control	10	423.55	$\frac{423.55}{10} = 42.355^{a} \pm 0.56$

Means sharing similar letter in a column are statistically non-significant (P>0.05); MEW of the harvested embryo from treated ECE; =39.745+38.575+38.08+37.72+37.185; =191.305/5; =38.261 grams; MEW of the harvested embryo from untreated ECE =37.28 grams; % Mean embryo weight (MEW) =MEW of the harvested embryo from treated ECE/MEW of the harvested embryo from untreated ECE * 100; % MEW =38.261 grams/37.28 grams * 100; =102.63%.

Micro-hemagglutination test: The results of micro-hemagglutination test were similar to that of spot hemagglutination test. The first two wells of microtitration plate containing NDV treated with undiluted stock solution and 1:2 of Neem bark extract, respectively inhibited the hemagglutination property of virus and thus showed antiviral activity. From 1:4 up to 1:512 dilutions the Neem bark extract did not inhibit the hemagglutination property of virus showing no antiviral activity of Neem bark extract. In first three wells, there was a clear change in the color of RBCs suspension from bright red to straw/serum like color with neither agglutination nor button formation. The results of micro-hemagglutination test are shown in Table 2, which describes that 1:4 dilution of the stock solution of Neem bark extract showed 75% reduction in hemagglutination titter of NDV whereas hemagglutination reduction was 100% in case of 1:8 dilution of Neem bark extract.

***In-ovo* antiviral and cytotoxicity assay:** Neem bark extract were used for the MNTC cytotoxicity assay. Estimated MNTC for Neem bark extract was 0.0783 mg/ml, whereas amantadine showed a MNTC of $0.9x10^{-3}$. Results of reduction in HA titer of NDV in Neem bark extract treated embryonated eggs are presented in Table 1.

The cytotoxicity was checked by performing cytotoxic assay, the test was performed in triplicate and results were averaged. The stock solution of Neem bark extract at 1:2 and 1:4 dilutions showed cytotoxicity to embryos in embryonated chicken eggs whereas no toxicity was observed at higher dilutions (Table 3).

The results presented in Table 3 showed the minute antiviral activity with less mortality percentage as compared to other groups but with severe lesions on embryo, which might be due to cytotoxicity of Neem barks extract. Statistically there was no significant difference in the antiviral effects of different concentrations of Neem bark extracts but the exposure time was a significant variable for cytotoxicity.

DISCUSSION

Present study was conducted to evaluate the antiviral activity of *Azadirachta indica* against ND virus. Findings of the current study are in line with the results of Li *et al.* (2005), which described concentration of Neem has direct relation with antiviral activity. The *in-vitro* activity was assessed by performing the spot assay and micro-hemagglutination test. The results are presented in Tables 1 and 2. In spot assay (Table 1), it was observed that NDV with 1st two dilutions 1:2 and 1:4 could inhibit the agglutination of chicken RBCs that shows its antiviral activity. Findings of the current investigation are same as the results of Chen *et al.* (2010), who performed similar work by using Curcumin, a natural compound in *Curcuma longa* that has antiviral properties against the enveloped viruses.

The results of micro-hemagglutination test shown in Table 2, describe that 1:4 dilution of the stock solution of Neem bark extract showed 75% reduction in hemagglutination titter of NDV whereas hemagglutination reduction was 100% in case of 1:8 dilution of Neem bark extract. These findings are in agreement with Elizondo-Gonzalez *et al.* (2012).

The cytotoxicity was checked by performing cytotoxic assay, the test was performed in triplicate and results were averaged. The stock solution of Neem bark extract at 1:2 and 1:4 dilutions showed cytotoxicity to embryos in embryonated chicken eggs whereas no toxicity was observed at higher dilutions (Table 3). Elizondo-Gonzalez *et al.* (2012) performed a similar study to assess cytotoxicity of Fucoida and Ribavirin against NDV and reported that Fucoidan exhibited antiviral activity against NDV La Sota, in which they reported viral inhibition in the early stages of infection (0–60 min post-infection). They also reported inhibition of viral penetration experiments with a wild-type NDV strain supported this result, as these experiments demonstrated a 48% decrease in viral infection as well as reduced HN protein expression. Ribavirin, which was used as an antiviral control, exhibited lower antiviral activity than fucoidan and high toxicity at active doses. In the fusion assays, the number of syncytia was significantly reduced (70% inhibition) when fucoidan was added before cleavage of the fusion protein, perhaps indicating a specific interaction between fucoidan and the F0 protein.

The results presented in Table 3 showed the minute antiviral activity with less mortality percentage as compared to other groups but with severe lesions on embryo, which might be due to cytotixicity of Neem barks extract. Statistically there was no significant difference in the antiviral effects of different concentrations of Neem bark extracts but the exposure time was a significant variable for cytoxicity. The findings of the present work are in line with the findings of Goebel *et al.* (2007) who conducted a similar study to check the *in-ovo* antiviral potential of *Synadenium glaucescens* against NDV.

Similarly, Liu and Yan (2009) reported *in vivo* efficacy of crude extracts from *Artemisia annul* L. against NDV in 9-11 day old embryonated chicken eggs. Significant effects of extracts were found on NDV replication.

The findings of the present study are in line with the results of Mabiki *et al*. (2013) and Claudia *et al*. (1996), who conducted a similar study to check the *in-ovo* antiviral potential of *Synadenium glaucescens* against NDV. Similarly, Liu and Yan (2009) reported *in vivo* efficacy of crude extracts from *Artemisia annul* L. against NDV in 9-11 day old embryonated chicken eggs. Significant effects of extracts were found on NDV replication. Sood *et al*., 2012 conducted a study on same line of action, they investigated the antiviral activity of *Eugenia jumbolana* crude extracts against avian influenza virus, results of current study are in accordance to that study.

Conclusions: In conclusion, Neem bark extract showed significant antiviral activity at higher concentrations during both *in-vitro* and *in-ovo* trials. Reduction in concentration is directly proportional to the reduction in antiviral activity and cytotoxicity. Neem bark extracts have shown promising efficacy as an antiviral agent and can be used as an effective antiviral alternate by minimizing its cytotoxic effects.

Authors contribution: MS and HWA executed the experiments and contributed in preparation of the manuscript. RZA, AR and BA analyzed the data and helped in manuscript preparation. All authors approved the final manuscript.

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