Original Research

Antiviral Effects of Garlic (*Allium sativum*) and Nilavembu (*Andrographis paniculata*) against Velogenic Strain of Newcastle Disease Virus: An *In ovo* Study**

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Abstract

The research work was conducted to evaluate antiviral effects of garlic (Allium sativum) and nilavembu (Andrographis paniculata) against Newcastle disease virus. Different concentrations (10 mg / ml, 50 mg / ml, 100 mg / ml and 200 mg / ml) of aqueous garlic extract, aqueous nilavembu extract, 50 % ethanol extract of nilavembu and 100 % ethanol extract of nilavembu were prepared. Lethality study of these extracts was carried out in embryonated chicken eggs (ECE). No lethality was observed up to 200 mg/ml and 100 mg / ml concentration of nilavembu and garlic extracts, respectively. Antiviral activity of different concentration of garlic and nilavembu extracts were added by virus neutralization test in ECE with velogenic strain of Newcastle disease virus (VNDV) in 8 group of birds. The group IX was kept as positive control by inoculating 0.2ml of VNDV and group X was kept as negative control by adding 0.2ml PBS. This study revealed that aqueous extract of nilavembu at higher concentration (100 mg / ml) and ethanol extract of nilavembu (10 mg / ml and 100 mg / ml) were found to have good antiviral property against Velogenic strain of Newcastle disease.

Key words: *Allium sativum, Andrographis paniculata,* Antiviral Activity, Newcastle Disease Virus

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Introduction

Newcastle disease (ND) is an economically important disease of poultry (Alexander and Senne, 2008). Newcastle disease virus (NDV) is an RNA virus from the genus Avulavirus of the family Paramyxoviridae (Mayo, 2002). NDV is grouped as lentogenic, mesogenic and velogenic pathotypes based on clinical signs, virulence and mortality of infected birds (Orsi *et al*., 2009). Lentogenic strains of NDV can produce mild respiratory infection, whereas mesogenic strains can cause low level mortality and neural signs and velogenic strains can result in severe mortality. Velogenic strains may be either neurotropic velogenic NDV or viscerotropic velogenic NDV (Huang *et al*., 2004; Piacenti *et al*., 2006). Outbreak of Newcastle disease has been regularly reported from different parts of the world even after vaccination. Hence, an alternate approach is necessary to control Newcastle disease. One such alternative strategy is the herbal therapy with the use of easily available and cheap herbs like garlic and nilavembu. The garlic and nilavembu which have antiviral property. Garlic is reported to have 33 sulfur compounds, several enzymes, 17 amino acids and minerals such as selenium (Newall *et al.*, 1996). Bioactive components of garlic like sulfur containing compounds (Alliin, Diallylsulfides and Allicin) may have antibacterial, antifungal, antiparasite, antiviral, antioxidant, antithrombotic, anti-cancerous and vasodilator characteristics (Amagase *et al.*, 2001). Nilavembu (*Andrographis paniculata*) is a member of the family of Acanthaceae, which has been used as a traditional herbal medicine in many parts of Asia and Europe (Jarukamjorn and Nemoto, 2008). Nilavembu (*A. paniculata*) contains andrographolide, neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide which are responsible for antiviral activity (Uttekar *et al*., 2012). Antiviral activity of nilavembu had been previously reported against Influenza (Chen *et al*., 2010), Herpes simplex virus-1 (Wiart *et al.*, 2005), HIV (Calabrese *et al*., 2000). Hence, the present study was carried out to evaluate their antiviral effect against Velogenic strain of Newcastle disease.

Materials and Methods

Preparation of Aqueous Garlic Extract

Aqueous garlic extract (stock) was prepared according to Shashikanth *et al*. (1981). Fresh garlic (50 g) was ground in a blender with 50 ml of distilled water. The mixture was filtered through a muslin cloth and centrifuged at 9000 rpm for 10 minutes. Then the supernatant was separated and filtered through 0.22 µm syringe filter. Different concentrations (10 mg / ml, 50 mg/ ml, 100 mg / ml and 200 mg / ml) of aqueous garlic extract were prepared by diluting the stock with appropriate volume of PBS.

Preparation of Aqueous and Ethanolic Extract of Nilavembu

Aqueous and ethanolic extract of nilavembu were prepared as per the procedure described by Sangeetha and Rajarajan, (2015). Fresh Nilavembu (*Andrographis paniculata*) leaves were washed in sterile distilled

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water to remove dust, dried in shade for 3 to 4 days and were ground finely in a blender and sieved through a muslin cloth. Dried powder (4 g) of nilavembu leaves were soaked in 10 ml of aqueous (100 %), aqueous ethanol (50 %: 50 %) and ethanol (100 %) and stored at - 4°C overnight. The extracts were filtered through gauze cloth to remove coarse particles and clarified by centrifugation at 9000 rpm for 10 min. The clarified extract was filtered using 0.22 µl Millipore filter and freeze dried in Christ® freeze drier. From the freeze dried powder different concentrations of nilavembu extracts were prepared.

Lethality Assay of Garlic and Nilavembu Extracts in Embryonated Chicken Eggs

Lethality of garlic and nilavembu extracts were assessed in 9 day old embryonated chicken eggs (ECE). Totally 51 ECE were divided to 17 groups in such a manner that each group consist of 3 ECE. Each concentration (10 mg / ml, 50 mg / ml, 100 mg / ml and 200 mg / ml) of aqueous garlic extract, aqueous nilavembu extract, 50 % ethanol extract of nilavembu and 100 % ethanol extract of nilavembu were inoculated into 3 ECE (G1-G16) via allantoic route. A total of three uninoculated ECE were kept as control (G17). The details were listed in Table 1. All the eggs were incubated in egg incubator at 37˚C and candled daily up to 7 days to check the viability of embryos.

Table 1: Experimental design for lethality assay

Group	Extract Inoculated	Concentration of Extract Inoculated (mg / ml)
I	Garlic aqueous extract	10
II		50
III		100
IV		200
V	Nilavembu aqueous extract	10
VI		50
VII		100
VIII		200
IX	Nilavembu 50 % ethanol extract	10
X		50
XI		100
XII		200
XIII	Nilavembu 100 % ethanol extract	10
XIV		50
XV		100
XVI		200
XVII	Control	

Virus

Velogenic strain of Newcastle disease virus (VNDV) isolated from a field outbreak with an ICPI (Intracerebral Pathogenicity Index) of 1.98 was obtained from the Central University Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai. 100 EID_{50} / 0.1 ml virus suspension was

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prepared by titrating in 9 days old embryonated chicken eggs via allantoic route by following Reed and Meunch (1938) method.

Embryonated Chicken Eggs (ECE)

Fifty one numbers of nine days old chicken eggs were obtained from Poultry Research station, Tamil Nadu Veterinary and Animal Sciences University, Chennai, whose parents were negative for serum antibody against NDV (SAN).

Estimation of Antiviral Efficacy of Garlic and Nilavembu by Virus Neutralization Study

Virus neutralization test was carried out in 9 days old embryonated SAN chicken eggs. Equal volume of VNDV (100 EID_{50} / 0.1 ml) and two different concentrations of (10 mg / ml and 100 mg / ml) of aqueous garlic extract, aqueous nilavembu extract, 50 % ethanol extract of nilavembu, 100 % ethanol extract of nilavembu were mixed and incubated at 37˚C for 1h for neutralization. Then 0.2 ml of each mixture was inoculated into 5 eggs. Five eggs are inoculated with 0.2 ml of VNDV (100 EID_{50} / 0.2 ml) alone and kept as positive control. For negative control five ECE were inoculated with 0.2 ml of PBS alone. All the inoculated eggs were incubated in an egg incubator at 37˚C and candled daily up to 7 days to check the viability of the embryo. After 7 days the eggs were chilled overnight at 4˚C; allantoic fluid was harvested from each egg and pooled sample of each group was subjected to Heamagglutination (HA) test as per OIE (2014) and embryonic lesions were also studied.

Results

Lethality Assay of Garlic and Nilavembu Extracts in Embryonated Chicken Eggs

In lethality assay, 100% embryo mortality was observed in Group IV (aqueous garlic extract 200 mg / ml) alone and no mortality were observed in other groups.

Antiviral Efficacy of Garlic and Nilavembu Extracts by Virus Neutralization Test (VNT)

The results of VNT viz. embryo mortality, HA titre and embryonic lesions were listed in Table 2 and Plate 1.

Discussion

Newcastle disease virus poses constant threat to the poultry industry. It is one among the highly contagious and economically important diseases of the poultry. Although regular vaccination has been followed to control Newcastle disease, outbreak of NDV has been reported even in vaccinated flocks (Al-Hadid *et al*., 2016). The alternative strategy to control Newcastle disease is prophylaxis with easily available herbs. Garlic and Nilavembu have been reported to have antiviral property (Lee *et al*., 2014 and Shojai *et al*., 2016) and are easily available and economical to use in poultry. Hence, the present study was carried out

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to evaluate their antiviral effect against Velogenic strain of Newcastle Disease Virus. Garlic is reported to have 33 sulfur compounds, several enzymes, 17 amino acids and minerals such as selenium (Newall *et al.*, 1996).

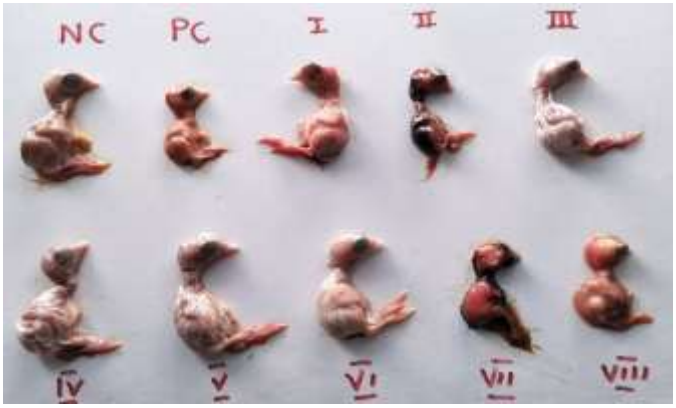

Plate 1: Embryonic lesions observed in Virus Neutralization test (VNT)
NC- Negative control; PC- Positive control; G1- Nilavembu aqueous extract100 mg / ml; G2- Nilavembu aqueous extract10 mg / ml; G3- Nilavembu 50% ethanol 100 mg/ ml; G4- Nilavembu 50% ethanol 10 mg/ ml; G5- Nilavembu 100% ethanol 100 mg / ml; G6- Nilavembu 100% ethanol 10 mg / ml; G7- Garlic aqueous extract 100 mg / ml and G8- Garlic aqueous extract 10 mg / ml.

Table 2: Antiviral efficacy of Garlic and Nilavembu

Group	No. of ECE inoculated	No. of embryos died	Mortality (%)	Embryonic lesion	HA titre (log 2)
(Nilavembu aqueous extract 100 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	-	-	-	-
(Nilavembu aqueous extract 10 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	2	40%	Haemorrhages on embryos	3
(Nilavembu 50 % ethanol extract 100 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	-	-	-	-
(Nilavembu 50 % ethanol extract 10 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	-	-	-	-
(Nilavembu 100 % ethanol extract 100 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	-	-	-	-
(Nilavembu 100 % ethanol extract 100 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	-	-	-	-
(Garlic aqueous extract 100 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	4	80%	Occipital & diffuse haemorrhage on embryos	7
(Garlic aqueous extract 10 mg / ml + 100 EID $_{50}$ / 0.1 ml of VNDV)	5	5	100%	Occipital & diffuse haemorrhage	7
(Positive control-100 EID$_{50}$ / 0.2 ml of VNDV)	5	5	100%	Occipital & diffuse haemorrhage	7
(Negative control-0.2 ml PBS)	5	-	-		

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The sulfur compounds found in fresh garlic appear to be nearly 1000 times more potent as antioxidants than crude and aged garlic extract (McCaleb, 1993). Zakaria (2003) reported that there was a loss of 15-29 % of antimicrobial activity of garlic when it stored in refrigerator for 6 days. Hence, aqueous garlic extract was prepared a fresh for this study. Nilavembu (*A. paniculata*) contains andrographolide, neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide which are responsible for antiviral activity (Uttekar *et al*., 2012). Antiviral activity of nilavembu had been previously reported aganist Influenza (Chen *et al*., 2010), Herpes simplex virus-1 (Wiart *et al*., 2005) and HIV (Calabrese *et al*., 2000).

To study the lethal effect of garlic and nilavembu on embryonated eggs, different concentration of garlic and nilavembu extracts were inoculated into 9 days old ECE in which garlic at a concentration of 200 mg / ml was found to have toxic effects in embryos and caused embryo mortality. Other groups didn't cause any embryo mortality and were nontoxic. Based on lethality assay results two different concentrations of extracts (low -10 mg / ml) and (high - 100 mg / ml) were chosen for virus neutralization study. In virus neutralization study, no mortality was observed in Group I, III, IV, V, VI and X and the same was 40, 80, 100 and 100 per cent in Group II, VII, VIII and IX, respectively. It was observed that aqueous extract of nilavembu at the concentration of 100 mg / ml and ethanol extract of nilavembu even at 10 mg / ml concentration were having good antiviral property. This correlates with the findings of Suriani *et al*. (2015) who reported antiviral efficacy of ethanolic extract of nilavembu even at a low concentration of 15 µg / ml. In order to evaluate the efficacy of aqueous extract of nilavembu, higher concentration than Suriani *et al*., (2015) was used in this study, so that aqueous extract can easily be prepared and used in the field condition. Antiviral effect of garlic against Infectious Bronchitis virus was reported by Shojai *et al*. (2016). On contrary, the present study showed no antiviral effect of garlic against velogenic strain of Newcastle disease virus. No HA activity was found in Group I, III, IV, V, VI and X. HA titre of 8, 128,128 and 128 were observed for Group II, VII, VIII and IX, respectively . The results are in accordance with findings of Al-Haldid *et al*. (2016), who reported HA titre of 3.0 to 5.6 for plant extracts of 20-80 % mortality in a virus neutralization study.

Conclusion

The results of this study confirmed the antiviral effect of nilavembu against NDV. Ethanolic extract of nilavembu is superior in antiviral activity than aqueous extract. Higher concentration (100 mg / ml) has always to be chosen when aqueous extract of nilavembu is used in the field.

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