Antiviral Efficacy of Neem, Garlic, and Ginger Extracts on Newcastle Disease Virus in Poultry: Impact of Concentration and Heat Treatment

ABSTRACT

Aims: Newcastle disease virus (NDV) is a paramyxovirus that causes significant mortality rates in poultry, often known as Newcastle disease or Ranikhet. This virus has the potential to inflict serious economic losses on farmers. As there is no effective therapy for NDV infection, the current research investigated the efficiency of medicinal plant extracts against this virus.

Place and Duration of Study: The experiment was conducted at the Department of Physiology, Biochemistry, and Pharmacology and the Department of Microbiology and Veterinary Public Health, Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh, during a period from January to April 2017.

Methodology:Plant samples (neem bark, neem leaf, garlic, and ginger) were collected from the medicinal garden of CVASU. Samples were ground,and 300 g of ground samples were mixed with distilled water in a 1:5 ratio.Mixtures were stirred for 1 hour by an electric stirrer (1000 rpm) and left overnight. All the mixtures were filtrated through Whatmanno. 1 filter paper separately. Finally,aqueous extracts were prepared from the filtrated sample with the help of a round bottom flask of a rotator vacuum evaporator.Then, the extracts were categorized into different groups to determine the possible effects of extract concentration and heat treatment on the antiviral potential of plant extracts. The plant extract was mixed with viable NDV (8 HA; field strain) and kept at 27.3 °C for 30 minutes. To determine virus inactivation, a 0.2-ml mixture was inoculated into nine-day-old embryonated chicken eggs and incubated. After 48 hours, the allantoic fluid was harvested, and a hemagglutination (HA) assay was performed to determine the virus HA titer.

Results: The antiviral effect of plant extract is described in terms of HA titers, specifically the geometric mean titer (GMT).The lower GMT titer value of the plant extract showed higher antiviral activity. However, Neem is more efficient against NDV than other extracts (garlic and ginger).The antiviral activities of these extracts can vary due to the concentration and heat treatment (autoclave) of the extracts. The antiviral potency of all plant extracts declines with decreasing concentration. Heat treatment significantly ($p = 0.02$) decreasesthe plant's extract antiviral efficacy.

Conclusion: This study suggests the potential use of common local medicinal plants to treat Newcastle disease in poultry, although active compounds of those plants have not yet been studied. Finally, these plants can be a promising source for developing antiviraldrugs against Newcastle disease.

Keywords:Newcastle disease, Anti-viral medicine, Medicinal plants, Aqueous extracts, Hemagglutination test.

1. INTRODUCTION

Newcastle disease virus (NDV) is a negative-sense single-stranded RNA virus under the family Paramyxoviridae (Mayo, 2002). It causes a severe disease in poultry named Newcastle disease, or Ranikhet. This disease remains a serious economic challenge to all segments of the poultry industry because of its contagious and mortality (0–100%) records (Chollom et al., 2012; Sulaiman et al., 2013), although mortality varies depending on the

pathotype of the virus. Medicinal plants are a rich source of natural compounds such as tannins, polyphenols, proanthocyanidins, sulfonamides, anti-adhesives, etc. that exhibit antiviral (Mukhtar et al., 2008; Gupta et al., 2014) and anti-inflammatory (Gupta et al., 2014) activities. Many reports showed that many indigenous communities used their herbal preparations for veterinary use (Madadgar et al., 2013). Like many other medicinal plants, Neem (*Azadirachta indica*) has been used in Ayurvedic medicine for more than 2000 years, and now it is being used in modern medicine, cosmetics, and pharmaceuticals as the global scenario is changing towards the use of nontoxic plant products. Its medicinal values come from the fruits, seeds, leaves, roots, and bark (Biswas et al., 2002). Various preparations of neem obtained from its different parts have been found to exert anti-bacterial, anti-viral, anti-malarial, anti-oxidant, anti-fungal, anti-mutagenic, anti-carcinogenic, contraceptive, and antiulcer activity (Subapryya and Nagini, 2005;Bonsu et al., 2012). Garlic has been an interesting plant for centuries as a medicinal panacea. A broad range of pathogenic organisms, including bacteria, fungi, protozoa, and viruses, are sensitive to fresh, crushed garlic (Mehrbod et al., 2009). *Zingiber officinale* (family Zingiberaceae), commonly known as ginger, is commonly used as an effective medicine against coughs and colds. It was also found effective against Newcastle disease virus during an *in vitro* experiment (Kikuzaki et al., 1991; Sharma and Gupta, 1998;Untari et al., 2022). However, since there is a lack of sufficient information about the antiviral potential (and factors affecting this potential) of neem leaves, neem bark, garlic, and ginger against the Newcastle disease virus, this study focused on finding out the effects of concentration and heat treatment (during autoclaving) on the anti-viral activity of these plant extracts. These plants had been used as traditional medicines by the natives for many years due to their antibacterial, antifungal, anti-allergic, anti-viral, and other important medicinal properties.

2. MATERIAL AND METHODS

2.1 Sample collection

The experiment was conducted at the Department of Physiology, Biochemistry, and Pharmacology and the Department of Microbiology and Veterinary Public Health, Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh, from January2017 to April 2017.

2.2 Virus sample

A previously isolated and stored (-80 °C) field strain of the Newcastle disease virus was collected from a repository of the Department of Microbiology and Veterinary Public Health, CVASU. After thawing, the virus sample was treated with an antibiotic (Gentamicin) to prevent bacterial contamination. Then the virus samples were subjected to a hemagglutination (HA) test to confirm the viability and concentration of the virus.

2.3 Plant samples

The bark and leaf of Neem (*Azardirachta indica*), Garlic (*Allium sativum*), and Ginger (*Zingiber officinale*) from the medicinal plant garden of Shahedul Alam Qudary Teaching Veterinary Hospital, CVASU, Bangladesh.

2.4 Plant extract preparation

Collected neem leaves, garlic, and ginger were thoroughly cleaned with water to remove dirt and unwanted materials. Then 300-gram (g) samples were ground using an electrical grinder. The ground samples (300 g) were mixed with distilled water at the ratio of 1:5 (sample: water) for neem leaf, garlic, and ginger (Iwalokun et al., 2004; Kwawukume et al., 2013; Gupta and Chaphalkar, 2015). In the case of neem bark, the collected barks were cut into small pieces and dried at room temperature (27°C) for several weeks. Then dried barks were pulverized using an electric grinder. Finally, 300 g of powder was mixed with distilled

water in a 1:10 ratio (Mahmood et al., 2017). Sample mixtures were stirred for 1 hour by an electric stirrer (1000 rpm) and left overnight. All the mixtures were filtrated through Whatmanno. 1 filter paper separately. Then 300 ml of filtrates from different samples were taken into a round bottom flask of a rotator vacuum evaporator and condensed by the evaporation of solvent from the filtrate in a water bath at 56.7°C for 3-5 hours. After the evaporation of solvent from the filtrate, the condensed extracts were preserved in a tightly corked-labeled bottle and stored at 4 °C.

2.5 Categorization of plant extracts

Each plant extract was broadly categorized into two groups: Group-I: extract without heat treatment, and Group-II: extract treated with heat at 121°C for 15 min. Then every group was further divided into three sub-groups by mixing PBS with concentrated plant extract, such as sub-group I: 100% extract (concentrated extract); sub-group II: 75% extract (three parts extract and one part PBS); and sub-group III: 50% extract (one part extract and one part PBS).

2.6 Toxicity test of samples

All types of plant extract were checked for any possible toxicity to chicken embryos by an embryonated egg inoculation assay. 0.2 ml of aqueous plant extract was inoculated in nine-day-old embryonated chicken eggs (5 eggs per concentration) collected from the Regional Poultry Farm (RPF), Chittagong. Then all eggs were incubated for 48 hours to check for embryo mortality.

2.7 Preparation of 8 HA unit virus

A virus sample (0.2 ml) was propagated in embryonated chicken eggs. After propagation, allantoic fluid, collected after 48 hours of incubation of eggs, was subjected to an HA test to determine 1 HA unit. Based on the Hemagglutination (HA) test result, an 8 HA unit virus concentration was prepared by mixing 15 ml of sterile PBS with 1 ml of allantoic fluid.

2.8 Embryonated eggs inoculation

Inoculums were prepared by properly mixing the plant extracts from all sub-groups with 8 HA units of the virus at a 1:1 (500 µl extract with 500 µl virus) ratio in an Eppendorf tube and were incubated at room temperature for 30 minutes. Five nine-day-old embryonated chicken eggs per concentration were inoculated with 0.2 ml of inoculum following standard procedure. The inoculated eggs were incubated for 48 hours. The eggs were candled after 24 hours of inoculation to check embryo mortality. After 48 hours of incubation, eggs were transferred to a chilling temperature and kept for 24 hours. About 10% control for both viruses and extracts wasmaintained during the whole procedure. After 24 hours of chilling, allantoic fluids were harvested from each egg using a sterile syringe. The fluid was taken into the falcon tube. Then the HA test was carried out using 1% chicken RBC, collected from a specific pathogen-free flock, to determine the virus titer in the allantoic fluid.

2.9 Statistical analysis

All data were entered into a spreadsheet in MS Excel 2010. The data were sorted, cleaned, and coded using the Excel program before exporting for the analysis of the Geometric mean titer (GMT). Finally, the GMT of HA was used to determine reduced HA titer (%) and the effect of two key factors-concentration and heat treatment, on the antiviral potential of plant extract.

3. RESULTS

3.1 Toxicity of Extract

In the toxicity testing assay, no plant extract caused mortality in chicken embryos within 48 hours of incubation (Table 1).

Table 1: Determination of toxicity of extracts in embryonated eggs

Plant extracts	Concentration (%)	Total no of eggs inoculated	Death observed in embryonated eggs			
			24 hours	48 hours	24 hours	48 hours
			without heat-treated plant extracts used		heat treated Plant extracts used	
Neem Leaf	100	20	A (0/5)	A(0/5)	A (0/5)	A(0/5)
	50	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	33	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
Neem Bark	100	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	50	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	33	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
Garlic	100	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	50	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	33	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
Ginger	100	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	50	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)
	33	20	A (0/5)	A (0/5)	A (0/5)	A (0/5)

A= Indicates all alive eggs after 48 hr inoculation of extract

3.2 Overall antiviral potential of extracts

The anti-viral effect (reduced HA) of plant extract was found to be variable when compared with the virus control group (Table2). For the NDV field strain, the geometric mean of HA titer (GMT) of the virus control group was 128 HA units.100%,50%, and 33% of aqueous extracts of neem bark from group-I (without autoclave) exhibited 0 HA titer (GMT), which indicates complete (100%) inactivation of the virus in the embryonated chicken egg assay. Group II (using an autoclave) had GMT values of 16, 64, and 84, which correspond to decreases in viral titer of 87.5%, 50%, and 34.3% at 100%, 50%, and 33% concentrations, respectively. On the other hand, ginger extract was found to be the least effective medicinal plant against the ND virus. It showed high GMT values (101.59, 111.43, and 111.43 for group-I, and 97, 111.43, and 128 for group-II) that reflect the weak anti-viral potential (20.6%, 12.9%, and 12.9% GMT reduction by sub-groups of groups-I; 24.21%, 12.9%, and 0% GMT reduction by sub-groups of group-II) of ginger against NDV. All neem leaf extract sub-groups of groups I showed similar anti-viral activity (GMT 64 and 50% GMT reduction) despite being different in concentration, although sub-groups of group II showed gradually decreased anti-viral activity due to dilution. The GMT values were 64, 84, and 111.43, reflecting 50%, 34.3%, and 12.9% virus titer reduction, respectively, for three sub-groups of neem leaf extract. Garlic extracts are good enough to reduce ND virus titers if they are not autoclaved. During this study, non-autoclaved extracts reduced 58% to 71.2% of the virus titers, whereas heat-treated (autoclaved) extracts reduced 0% to 43.3% only.

Table 2: HA activity of Plant extract against NDV in embryonated eggs

Concentration	Plants	No of	HA titer of virus treated	Control	Reduced HA (%)

	Extract	eggs (n)	with extracts Geometric mean-titer (GMT)		GMT	=(*C-*E)/*C	
			Without heat treated	Heat treated		Without heat treated	Heat treated
100% (1:1)	Neem leaf	10	64	64	128	50	50
	Neem Bark	10	0	16		100	87.5
	Garlic	10	36.75	84		71.2	34.3
	Ginger	10	101.59	97		20.6	24.21
50% (1:2)	Neem leaf	10	64	84		50	34.3
	Neem Bark	10	0	64		100	50
	Garlic	10	45.25	97		64.6	24.2
	Ginger	10	111.43	111.43		12.9	12.9
33% (1:3)	Neem leaf	10	64	111.43		50	12.9
	Neem Bark	10	0	84		100	34.3
	Garlic	10	53.81	128		58	0
	Ginger	10	111.43	128		12.9	0

*Haemagglutination titer of virus control group,

*Haemagglutination titer of extracts treated embryonated group eggs

However, the antiviral activity of garlic also varied due to dilution (GMT 36.5, 45.25 and 53.81 in group-I; 84, 97 and 128 in group-II).

3.3 Effect of concentration and heat treatment on the antiviral potential of plant extract

There was a favorable correlation found between plant extract concentration and antiviral activity.

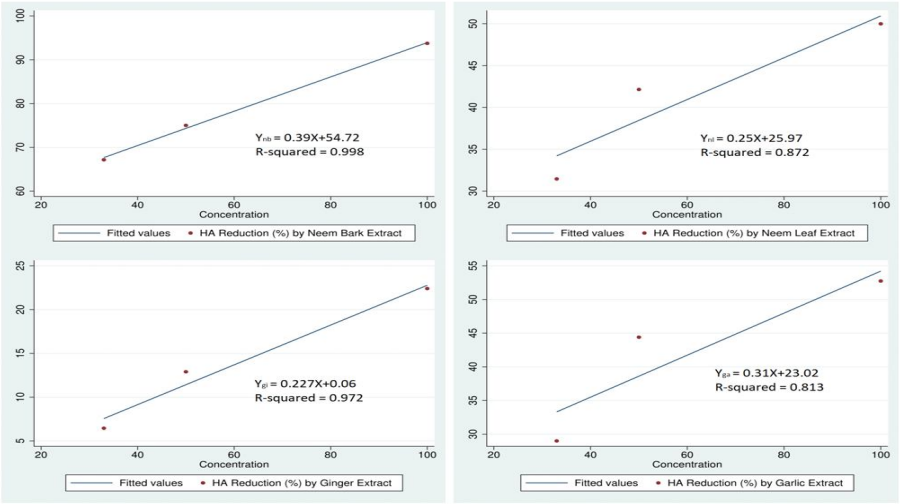

Figure 1: Regression lines reflecting effects of concentration of plant extracts on their anti-viral potential

The antiviral potential of all plant extracts decreases if the concentration of extract decreases. The regression analysis shows that concentration adversely affects the antiviral activity of neem bark (R2=0.998; slope=0.39), Neem leaf (R2=0.872; slope=0.25), Garlic (R2=0.813; slope=0.31) and Ginger (R2=0.972; slope=0.23) extracts (Figure 1). On the other hand, heat treatment has a negative effect on antiviral potential of plant extract (Figure 2).Due to autoclaving, the antiviral properties of plants decrease significantly (p=0.02).

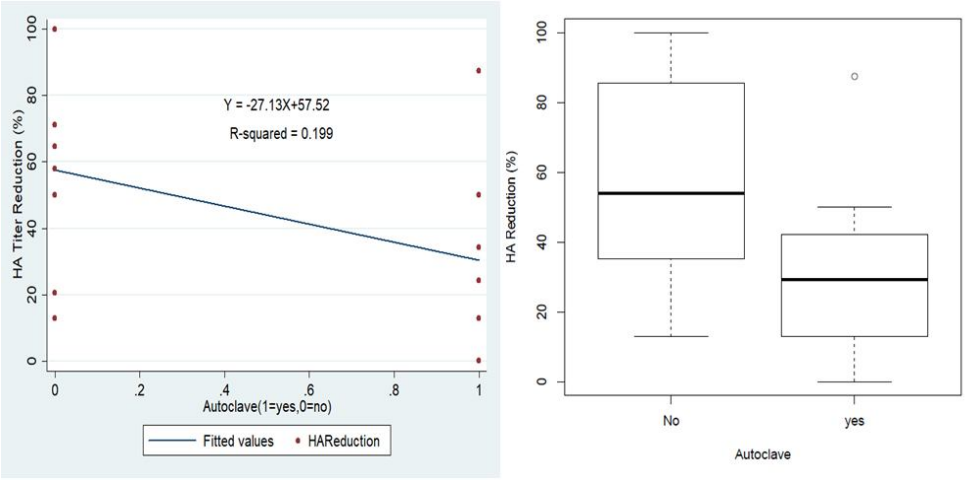

Figure 2: Regression line and box plot reflecting the effect of heat treatment (during autoclaving) on the antiviral activity of studied medicinal plant' extracts

However, the effect of concentration on the virus titer reduction ability of plant extract is not modified by heat treatment (Figure 3).

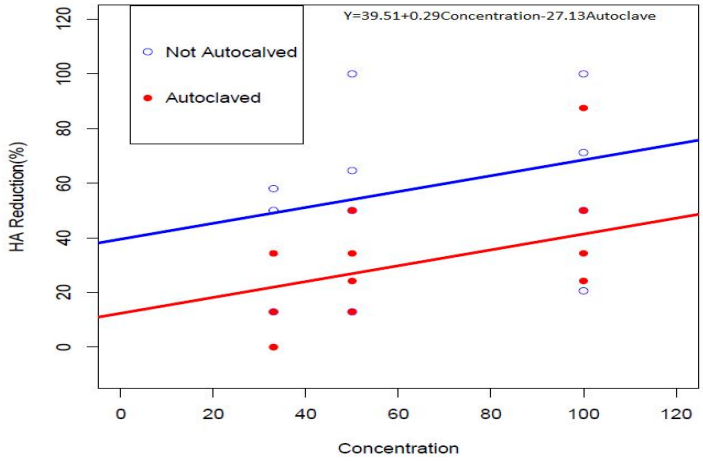

Figure 3: Combined effect of concentration and heat treatment on anti-viral activity of studied medicinal plants

4. DISCUSSION

Medicinal plants are the ultimate source for treating living organisms in humans and animals suffering from infectious and non-infectious diseases (Arunkumar and Muthuselvam, 2009). In this study, four aqueous extracts of three plant species, neem (Azardirachta indica), garlic (Allium sativum), and ginger (Zingiber officinale), were prepared and tested against NDV,

which causes economic losses to the poultry industry across the world. In this study, the antiviral impact of plant extract is described in terms of HA titers, especially the geometric mean titer (GMT). The lower the GMT titer value, the stronger the antiviral effect of plant extract, which was also described in another study by Sulaiman et al. (2011), Ong et al. (2014), and Mahmood et al. (2017). In this research, all the studied plants were found to be non-toxic to the chicken embryo, and their antiviral activities against NDV can vary due to differences in species, concentration, and heat treatment. These findings prove the previous research findings (Keqiang and van Bruggen, 2001; Mahmood et al., 2017). Neem is comparatively more effective against NDV compared to other plant extracts; this may be due to the difference in their phytochemical properties (Song et al., 1997; Parida et al., 2002). Without autoclaving, the use of neem extracts (both leaf and bark) shows almost similar effectiveness against NDV, irrespective of extract concentration. But after autoclaving, the antiviral activities of neem extract decrease with the decrease in extract concentration (Sulaiman et al., 2011; Ong et al., 2014; Mahmood et al., 2017). This phenomenon indicates the negative effect of heat treatment on the antiviral potential of neem plant extracts, which may happen due to the breakdown or degeneration of biochemical compounds (such as nimbidin, sodium nimbidate, epicatechin, catechin, etc.) that trigger antiviral activity (Biswas et al., 2002). Despite being affected by heat and concentration, the neem plant can be an incredible source of anti-viral therapeutics against NDV, as it also plays an important role in strengthening the immune system and inactivating viruses effectively (Sadekar et al., 1998; Awolu et al., 2013; Elbasuni et al., 2023). Garlic is a rich source of allicin and ajoene (Ankri and Mirel, 1999). Previously, garlic was found effective against infectious bronchitis virus during propagation in embryonated chicken eggs (Shojai et al., 2016). Besides, it can significantly increase the antibody titer against the ND and avian influenza viruses in poultry (Eid and Iraqi, 2014). This study found that garlic can also inactivate NDV; however, the extract's concentration and heat treatment have a major impact on its antiviral activity. Ginger, a source of different phenolic derivatives such as gingerol, paradole, bisabolene, zingerone, etc., has been used as an anti-viral agent in humans and animals for many years. Gupta and Chalkar (2015) described the anti-inflammatory and anti-viral potential against NDV of garlic. During this research, garlic was found to be able to inactivate NDV, but to a narrow extent. However, interestingly, the anti-viral activity of undiluted extract may be increased due to heat treatment. Due to the presence of gingerol, paradole, bisabolene, zingerone, zingiberol, and 6-shogaol, ginger (Zingiber officinale) can act as an anti-inflammatory and anti-viral agent. In previous research, ginger extracts were found effective against NDV (Mishra et al., 2012; Gupta and Chaphalkar, 2015), but in this study, ginger showed a lower antiviral effect against NDV, which may be due to the species differences of ginger and/or strain differences of virus used in these studies. Furthermore, the antiviral activity of ginger is conversely related to the dilution and autoclaving of the extract.

5. CONCLUSION

The aqueous extracts of all studied plants are effective against the Newcastle disease virus of poultry, although neems-bark extract is likely the most effective among the plant extracts. Heat treatment can affect the antiviral potential of medicinal plant extracts, which is worse in the case of garlic extract. Besides, theconcentration of extract also has a positive relationship with the antiviral activities of studied plants. Although the present study has not studied details, especially active compounds, of the studied plants, it unveils the possibility of using common local medicinal plants to treat Newcastle disease in poultry.

STUDY LIMITATIONS AND FUTURE DIRECTIONS

This study focused solely on the antiviral effectiveness of neem, garlic, and ginger aqueous extracts in embryonic eggs against NDV. The current study did not investigate details, particularly the active components of the studied plants. So further study is needed to

identify the active elements of these plants and their potential impacts on live birds. It will aid with the development of novel antiviral medications for Newcastle disease.

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COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTIONS

Mohammad Mahbub Hasan - Conceptualization, Methodology, Data curation, Formal analysis, writing, editing, Abdul Ahad- Conceptualization, Supervision, administration.

ETHICAL APPROVAL

Ethical Approval (CVASU/Dir(R&E) EC/2022/435(1)/7) was taken from the Ethical Approval committee of the Director of Research of Extension, Chittagong Veterinary & Animal Sciences
University.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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