American Journal of Agricultural and Biological Sciences

Original Research Paper

Evaluation of Antiviral Activity of Different Medicinal Plants against Newcastle Disease Virus

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Article history
Received: 28-05-2016
Revised: 12-10-2016
Accepted: 27-10-2016

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Abstract: Newcastle Disease (ND) is a highly contagious viral disease that has a tremendous negative impact on the poultry industry worldwide. Plant extracts were prepared from five different medicinal plants and applied against Newcastle Disease Virus (NDV) to evaluate the antiviral replication in Specific Pathogen-Free (SPF) chicken embryos. Three dilutions from each plant extract were prepared and mixed with a fixed titer (10^4 EID_{50}/mL) of virulent NDV strain Herts 33. The mix was inoculated into nine-day-old SPF chicken embryos that were monitored for five days. Real-time PCR and hemagglutination tests were conducted to evaluate the activity of NDV and its viral RNA titer. Some plant extracts showed a complete inhibition of NDV evidenced by the absence of embryo deaths, the absence of HA titer and viral RNA in the allantoic fluid. These plant extracts were from *Moringa peregrina* (leaves), *Acacia cyanophylla* (leaves), *Eucalyptus camaldulensis* (fruits) and *Pistacia atlantica* (leaves and stems). Other plant extracts showed partial inhibition of NDV, such as *Ceratonia siliqua* (leaves) and *Eucalyptus camaldulensis* (leaves). This experiment shows the potential of using medicinal plants as antiviral agents.

Keywords: Antiviral Activity, Medicinal Plants, Newcastle Disease Virus

Introduction

Newcastle Disease, caused by Newcastle Disease Virus (NDV), is a serious threat to the global poultry industry due to its high mortality rate. This is why outbreaks of virulent NDV require an immediate notification to the Office of International Epizootes (OIE) (Alexander, 2000). NDV is a negative-sense, single-stranded RNA virus of the family Paramyxoviridae. It is classified as an avian paramyxovirus 1 (APMV-1) (Taylor *et al*., 1990; De Leeuw and Peeters, 1999; Alexander, 2000). The NDV genome contains six major genes: Nucleocapsid, phosphoprotein, Matrix (M), Fusion (F), hemagglutinin-neuraminidase and RNA polymerase (De Leeuw and Peeters, 1999; Alexander, 2000). NDV isolates are classified into one of three pathotypes based on their pathogenicity: Lentogenic, mesogenic and velogenic (Alexander, 2000). This classification is confirmed by the presence of a cleavage site between amino acids 112-117 of NDV fusion protein. However, recent data suggest that this classification needs to be conducted not only by virus sequence analysis, but also by *in vivo* pathogenicity tests. These tests are required to demonstrate the clinical virulence of NDV as measured by the intracerebral pathogenicity index and the mean death time (Yu *et al*., 2001).

Vaccination is the main protector against NDV infection. However, different strategies are needed to either prevent the replication of NDV or to decrease its drastic effects on an infected flock (Dortmans *et al*., 2012; Miller *et al*., 2013). One of those strategies is investigating the antiviral activity of medicinal plant extracts against NDV infection (Waihenya *et al*., 2002). In fact, farmers mix medicinal plants with the poultry feed to increase the possibility of obtaining healthy chickens. In this study, I have selected five medicinal plants to test their antiviral activity against NDV. According to my knowledge, this is the first report of investigating the antiviral activity of NDV using plant materials of *Moringa peregrina, Pistacia atlantica, Eucalyptus camaldulensis, Acacia cyanophylla and Ceratonia siliqua*.

Moringa peregrina belongs to a flowering plant family called Moringaceae. This family has only one genus, called *Moringa*, which contains fourteen species

Science Publications

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from different tropical and subtropical regions (Dehshahri *et al*., 2012; Al Khateeb *et al*., 2013). Species of this family are distributed from the Dead Sea to southern Arabia and northern Somalia (Carvalho and Renner, 2012). *Moringa peregrina* is a good source of proteins, vitamins A, C and B, minerals and calcium. This is why leaves and pods are used as livestock feed (Asghari *et al*., 2015). The active biological ingredients of *Moringa peregrina* show anti-hyperglycemic and cytotoxic activity (El-Alfy *et al*., 2011).

Pistacia atlantica is a deciduous plant that belongs to Anacardiaceae family, which comprises about 600 species (Bozorgi *et al*., 2013). This plant is widely distributed worldwide, particularly in the Mediterranean region (Bozorgi *et al*., 2013). In folk medicine, *Pistacia atlantica* is used in the treatment of several diseases, such as; hypertension, eczema, kidney stones, cough and asthma (Tohidi *et al*., 2011; Oran and Al-Eisawi, 2015). In fact, *Pistacia* species demonstrates antioxidant, antimicrobial, anti-inflammatory, anti-diarrheal, anti-ulcer and hypoglycemic activity (Benhammou *et al*., 2007; 2008). *Pistacia* is rich in triterpenoids, phenolic compounds, fatty acids and sterols (Bozorgi *et al*., 2013). Recently, it was shown that essential oil of *Pistacia atlantica* has many different compounds, such as myrcene, limonene and sabinene (Bozorgi *et al*., 2013).

Eucalyptus camaldulensis is an evergreen tree that grows in a wide range of climatic conditions ranging from high rainfall to extreme drought. The essential oil of *Eucalyptus camaldulensis* leaves contains bioactive compounds that have antibacterial, anti-inflammatory, insecticidal, antioxidative and antiradical activity (Cheng *et al*., 2009; Silva *et al*., 2010; Huang *et al*., 2015). In folk medicine, *Eucalyptus* is used to combat upper respiratory tract infections and as an antiseptic drug (Silva *et al*., 2003).

Acacia cyanophylla evergreen plant belongs to Fabaceae family. The *Acacia* genus contains more than 1,030 species that are distributed in the sub-arid and arid portions of the world (Nasri *et al*., 2013). *Acacia cyanophylla* has antimicrobial (Ayeb-Zakhama *et al*., 2015), hypoglycemic (Wadood *et al*., 1989) and anti-inflammatory activity (Al-Mustafa and Dafallah, 2000). It has high level of tannins, phenolic compounds, carotenoids, tocopherols and sterols (Ben Salem *et al*., 1997; Nasri *et al*., 2013).

Ceratonia siliqua is an evergreen tree that belongs to Fabaceae family. This tree is characterized as slow-growing and adapted to drought conditions (Oliveira *et al*., 2011). The production of *Ceratonia* pods is estimated to be more than 300 tons per year, from about 200,000 hectares planted worldwide (Makris and Kefalas, 2004). In folk medicine, the plant is used to treat infant diarrhea (Loeb *et al*., 1989), cough and as an anti-diuretic agent (Oran and Al-Eisawi, 2015). The leaves and pulp have antimicrobial (Kivack *et al*., 2002), antioxidant (Papagiannopoulos *et al*., 2004) and anti-proliferative activity (Corsi *et al*., 2002). In fact, *Ceratonia silique* pod is rich in sugars, tannins, dietary fiber and polyphenols (Papagiannopoulos *et al*., 2004).

Using herbal extracts along with the NDV vaccine can enhance both cell-mediated (proliferation assay) and humoral immunity (increase in the anti-NDV antibodies titer) (Kong *et al*., 2004; 2006). Therefore, in this study, the use of some medicinal plants as anti-NDV was investigated.

Materials and Methods

Preparation of Plant Extracts and Newcastle Disease Virus

Five medicinal plants were collected and air-dried at room temperature. Different plant parts were used: The leaves of *Moringa peregrina,* the stem and the leaves of *Pistacia atlantica,* the fruits and the leaves of *Eucalyptus camaldulensis*, the leaves of *Acacia cyanophylla* and the leaves of *Ceratonia siliqua*.

Plant materials were ground to a fine powder. Plant ethanol extracts were prepared by adding absolute ethanol to plant material 1:2 (w/v). The suspension was incubated for 72 h at room temperature with gentle shaking. The plant suspension was filtered using Whatman paper (No. 1). The filtrate was concentrated using a rotary evaporator to remove the ethanol. The plant extract was suspended by adding 5.0 mL dimethyl sulfoxide (DMSO) to 0.5 g plant extract. Three concentrations of plant extracts were prepared: 500, 250 and 50 μg mL^{-1}. These dilutions were mixed with Herts 33 strain of NDV in a titer of 10^4 EID_{50}/mL and used in the *in ovo* experiment. 100% DMSO (Sigma. USA) was used to prepare all concentrations. DMSO has no effect on NDV activity (Bakari *et al*., 2012).

In ovo anti-Newcastle Disease Virus Experiment

Nine-day-old SPF chicken embryos were bought from a local hatchery. Three dilutions of each plant crude extract (500, 250 and 50 μg mL^{-1}) were mixed 1:9 with virulent NDV Herts 33 strain (10^4 EID_{50}/mL) and maintained at room temperature for one hour. After incubation, 100 μL of the plant-virus mixture was inoculated into the allantoic cavity. 100% DMSO was used to prepare all the dilutions. The final DMSO concentration used *in vivo* was 10%.

SPF eggs were incubated and monitored for five days. Embryo mortality was recorded daily. The allantoic fluid was harvested from dead and living embryos and then stored at -80°C. The positive control used for this experiment was NDV Herts 33 strain and the negative control was 1x Phosphate-Buffered Saline (PBS).

Hemagglutination (HA) Titration for Newcastle Disease Virus

A standard hemagglutination test was conducted on the harvested allantoic fluid to calculate NDV HA

titer for each sample. 25 µL of PBS was added into each well of a V-96 well plate. The same volume of inactive NDV was added to the first well. A two-fold dilution of 25 µL of the virus was prepared for each well of the whole plate. 25 µL of 1% (v/v) chicken RBCs was added to each well. Then, the solution was mixed gently in each well. The plate was incubated for 40 min at room temperature. Negative NDV chicken serum and positive NDV chicken serum were used as controls.

Viral RNA Isolation and Quantitative Real-time PCR Analysis

Allantoic fluids from dead and living SPF eggs were collected for viral RNA extraction to conduct a Real-time PCR experiment. Total RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Germany), according to the kit manufacturer's instructions. RNA was evaluated quantitatively and qualitatively using a spectrophotometer. The QuantiFast Probe RT kit was used (Qiagen, Germany) for the real-time PCR experiment. Primers and probe sequences for NDV detection were constructed according to (Farkas *et al*., 2009). The sequences are: NDVNP-F: TCTCTTATGCTCCCACTCTCAAGT, NDVNP: R CGATCTCAAGAACAGCCAGTGT, probe: CCTTGCAGGGAAACAG. Real-time PCR reaction volumes and conditions were applied as mentioned in (Farkas *et al*., 2009). Proper controls were included in each run. Results of the real-time PCR were expressed as a threshold cycle (Ct). A low Ct value indicates high viral RNA concentration, whereas a high Ct value indicates low viral RNA concentration in the sample.

Results

The mortality of the SPF embryos was recorded for five days post-inoculation with the plant-virus mixture (Table 1). Some plant extracts showed complete inhibition of NDV. For instance, high and medium concentrations (500 and 250 µg mL^{-1}) of *Moringa peregrina* leaves extract had a complete inhibition of the NDV replication with zero mortality. However, when low concentration (50 µg mL^{-1}) of *Moringa peregrina* leaves extract was used, the mortality was 40%. Similar results were obtained using *Eucalyptus camaldulensis* fruits, which showed complete NDV inhibition at low and medium plant extract concentrations. However, 20% mortality was recorded when a high *Eucalyptus camaldulensis* fruits extract concentration (500 µg mL^{-1}) was used. *Ceratonia siliqua* leaves extract did not show complete NDV inhibition at any of the plant extract concentrations. High, medium and low *Ceratonia siliqua* leaves extract concentrations (500, 250 and 50 µg mL^{-1}) showed 20, 40 and 80% mortality, respectively. This demonstrates a dose-dependent pattern of plant extract and mortality.

An optimal concentration of plant extract was able to inhibit the replication of NDV inside the embryonated SPF eggs. For instance, medium concentration (250 µg mL^{-1}) of *Pistacia atlantica* (leaves and stem), *Eucalyptus camaldulensis* fruits and *Acacia cyanophylla* leaves extracts showed zero mortality.

To investigate the activity of NDV in the collected allantoic fluids, the HA titer was calculated. HA titer was not detectable for plant extract treatments with complete NDV inhibition and zero mortality. However, the mean NDV HA titer ranged from 3.0 to 5.6 for plant extracts of 20 to 80% mortality (Table 2). Moreover, the threshold cycle (Ct) value was not detected for the plant extracts that yielded zero mortality. This indicates the absence of NDV viral RNA in SPF eggs. However, Ct values ranged from 27.5 to 34.1 for the plant extracts that yielded some mortality. This indicates the presence of NDV viral RNA in SPF eggs. Real-time PCR results match well with the NDV HA titer results (Table 2). A correlation coefficient (R^2) value was calculated as 0.99 for the real-time PCR experiment (Fig. 1).

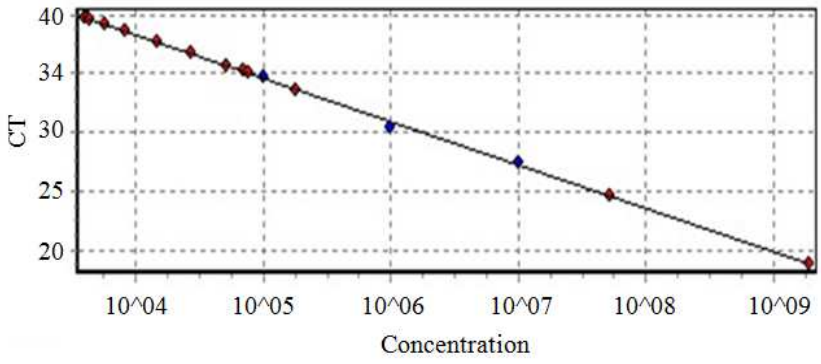

Fig. 1. Real-time PCR correlation coefficient (R^2). R^2 value was 0.99065 for the quantification of the viral RNA titer of NDV. This shows that the real-time experiment was conducted with a high degree of accuracy

Table 1. Mortality percentage of *in ovo* anti-Newcastle disease experiment for different plant extract treatments

Plant part	Leaves					Fruits	Stem
Plant extract concentration[a]	*M. peregrina*	*P. atlantica*	*E. camaldulensis*	*A. cyanophylla*	*C. siliqua*	*E. camaldulensis*	*P. atlantica*
500 μg mL^{-1}	0/5:0%*	1/5:20%	3/5:60%	2/5:40%	1/5:20%	1/5:20%	1/5:20%
250 μg mL^{-1}	0/5:0%	0/5:0%	2/5:40%	0/5:0%	2/5:40%	0/5:0%	0/5:0%
50 μg mL^{-1}	2/5:40%	1/5:20%	2/5:40%	1/5:20%	4/5:80%	0/5:0%	3/5:60%

* Mortality Percentage (0/5: zero%, 5/5: 100%)

Table 2. Calculated mean HA titer and threshold cycle (Ct) for the different plant extract treatments. HA titer mean is inversely proportional to the amount of viral RNA

Plant part	Leaves															Fruits			Stem			
Plant extract	*M. peregrina*			*P. atlantica*			*E. camaldulensis*			*A. cyanophylla*			*C. siliqua*			*E. camaldulensis*			*P. atlantica*			
concentration[a]	500	250	50	500	250	50	500	250	50	500	250	50	500	250	50	500	250	50	500	250	50	
HA titer (Mean Log^2)	ND[c]	ND	5.3	3	ND	4	5	5	4.3	3	ND	3	3.6	4.3	5.6	3.3	ND	ND	3.3	ND	5.6	
Viral RNA titer in Ct[b]	ND	ND	29.4	31.4	ND	31.9	28.6	31.6	30	32.4	34.1	32.3	31.7	33	27.5	29.4	ND	ND	33	ND	29	

a Concentration in (μg/mL)
b Threshold Cycle (mean)
c ND: Not Detectable

Discussion

Newcastle Disease Virus remains a continuous threat to the poultry industry worldwide due to the capability of the virulent strains to cause high mortality. Vaccination against NDV has a crucial role in protecting poultry from the virulent strains of NDV (Miller *et al*., 2013). However, the vaccine could take a long time to initiate the protective immune system. Therefore, different strategies to either prevent the replication of NDV or to decrease its drastic impact on infected flocks are needed (Park *et al*., 2014). One of those potential strategies is to use medicinal plant extracts as antiviral agents against NDV either in cell culture or chicken embryo systems (Waihenya *et al*., 2002). Therefore, in this study, we examined the antiviral activity of five medicinal plants on the inhibition of NDV replication inside SPF-embryonated eggs. These plants were: *Moringa peregrina, Pistacia atlantica, Eucalyptus camaldulensis, Acacia cyanophylla* and *Ceratonia silique*. These plants were selected due to their potential as antiviral agents since their antimicrobial activities have been reported (Cimanga *et al*., 2002; Kivack *et al*., 2002; Ayeb-Zakhama *et al*., 2015; Roozegar *et al*., 2016). Moreover, these plants are distributed worldwide. This makes them available to farmers with low cost. The most dominant part of these plants are the leaves. This is why leaves were selected for this study. However, the essential oil of *E. camaldulensis* fruits was reported to have an antimicrobial activity (Knezevic *et al*., 2016). Therefore, *E. camaldulensis* fruits were selected for this study. *P. atlantica* stem was selected to find out if the stem has a different effect than the leaves.

Responses to the different treatments using medicinal plant extracts to protect chicken embryos against NDV can be classified into four groups. Group I is represented by *Moringa peregrina* leaves and *Eucalyptus camaldulensis* fruits, where some concentrations of plant extracts showed complete inhibition of the virus with zero mortality. The *Moringa peregrina* leaves extract required moderate to high concentrations to achieve complete inhibition and zero mortality. However, the *Eucalyptus camaldulensis* fruits extract showed complete inhibition of the virus and zero mortality at low and moderate plant material concentrations. When the *Eucalyptus camaldulensis* fruits extract was applied at high concentration, a 20% mortality was measured. This could be due to reaching some level of toxicity to the chicken embryo.

Group II is represented by *Pistacia atlantica* (stem and leaves) and *Acacia cyanophylla* leaves, where a medium plant extract concentration was able to provide complete inhibition of the virus, whereas low and high concentrations of the plant extract showed some percentage of mortality. This means that low plant extract concentrations were not enough to provide complete protection from the virus, while high plant extract concentrations were toxic to the chicken embryo. Stem and leaves had similar effect. This could indicate that the active ingredient that is responsible of the viral inhibition and the toxicity exists into both parts.

Group III is represented by *Ceratonia siliqua* leaves, where a dependent-dose plant extract response is observed as the degree of virus inhibition. However, complete inhibition of viral activity was not obtained, suggesting the need to apply higher concentrations of the plant extract.

Group IV is represented by *Eucalyptus camaldulensis* leaves, where no complete inhibition of the viral activity was detected using any concentration of the plant material. However, at higher concentrations

of the extract, the same or higher mortality was measured. This is due to increasing toxicity when using higher concentrations of plant material. The virus did not affect the mortality since similar RNA viral amounts were detected.

These results correlate almost perfectly in most of the treatments with the detected viral RNA in each sample using real-time PCR technique. Plant extract concentrations yielding complete inhibition of the viral activity had no NDV viral RNA. However, plant extract concentrations with partial inhibition of the viral activity had low NDV viral RNA. Here, I showed that some of these medicinal plant extracts were able to provide complete protection for the SPF embryos against NDV with zero mortality.

Farmers are recommended to use plant extracts as one of the protective practices to inhibit NDV. The recommended doses are 250 μg mL^{-1} of *Moringa peregrina* leaves, 250 μg mL^{-1} of *Pistacia atlantica* leaves or stem and 50 μg mL^{-1} of *Eucalyptus camaldulensis* fruit. The results of this study confirm those of other studies that used medicinal plant extracts as antiviral agents against NDV. For instance, the inhibitory concentration of *Adansonia digitata* bark or *Psidium guajava* leaves was 200-250 mg mL^{-1} against NDV (Sulaiman *et al*., 2011; Chollom *et al*., 2012a). Moreover, the inhibitory concentrations of fruit pulp and leaves of *Momordica balsamina* was 10-20 mg mL^{-1} (Chollom *et al*., 2012b). Other medicinal plants showed stronger inhibitory effects, such as leaves, roots and stem bark of *Commiphora swynnertonii,* which had an inhibitory concentration of 500 μg mL^{-1} against NDV (Bakari *et al*., 2013). Therefore, *Moringa peregrina* leaves extract and *Pistacia atlantica* leaves and stem extracts have strong inhibitory effects against NDV, while *Eucalyptus camaldulensis* fruit extract has a very strong inhibitory effect against NDV.

Further experiments need to be conducted to test the use of those plant extracts that showed complete inhibition of the viral activity and zero mortality as supplements to broilers' diets to protect them against NDV. Moreover, further studies will include the precise dose to be used for inhibition within the range of plant extract concentrations that were tested in this study.

Conclusion

Ethanolic extracts of *Moringa peregrina* leaves, *Acacia cyanophylla* leaves, *Eucalyptus camaldulensis* fruits and *Pistacia atlantica* (leaves and stem) showed complete inhibition of NDV without causing death of the chicken embryo. The response of the viral-infected chicken embryos is different depending on the species of the medicinal plant, plant part and the plant material concentration applied. Some plant extracts show a dose-dependent relationship with the degree of the virus inhibition, whereas other plant extracts showed some toxicity on the chicken embryo. This study shows the potential of using medicinal plant extracts as an antiviral agent to protect broilers from NDV.

Acknowledgement

I would like to thank Royal Botanic Garden of Jordan and the National Center for Agricultural Research and Extension for providing the plant materials. I also would like to acknowledge Dr. Mustafa Ababneh for facilitating the molecular work in his lab at Jordan University of Science and Technology, Irbid, Jordan.

Funding Information

I would like to thank Hamdi Mango Research Center for Scientific Research, The University of Jordan, Amman, Jordan, for providing the fund for this project.

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