

Antiviral activity of green silver nanoparticles produced using aqueous buds extract of *Syzygium aromaticum*

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Abstract: In the present study, we synthesized silver (Ag) nanoparticles using aqueous extracts of clove (*Syzygium aromaticum*) (SAE). This synthesis of green silver nanoparticles (AgNP) was a novel and effectual tool against the Newcastle Viral Disease (NDV). *Syzygium aromaticum* extract was used as reducing and stabilizing agent for synthesis of silver nanoparticles. AgNP were characterized using diversity of biophysical methods inclusive of Fourier transform infrared spectroscopy (FTIR), UV-VIS spectroscopy and Transmission electron microscopy (TEM) for morphology and size. Furthermore, XRD analysis confirmed the crystalline nature of the particles. In current investigations, the antiviral activity of clove buds silver nanoparticles was inspected *in-vitro* and *in-ovo*. Embryonated chicken eggs were used to perform the cytotoxicity assay of the clove extract silver nanoparticles (CESN). CESN showed *in vitro* antiviral activity against NDV in embryonated eggs.

Keywords: Embryonated, cytotoxic, *Syzygium aromaticum*, nanoparticles, antiviral.

INTRODUCTION

ND is an infectious avian disease that affects numerous house-hold and wild bird species (Omer *et al.*, 2014). Ranikhait is the common name of Newcastle Disease (ND) in Indo-Pak (Narayanan *et al.*, 2010) which is spread by Newcastle Virus (NV). ND is responsible for big economic loss to the commercial poultry farmers throughout the world. Extreme outbreak of ND reasoned by avian paramyxovirus serotype-1 (APMV 1) serotype, ensued in Jallo Wildlife Park, Lahore in 2012. 190 peacocks died in a week having mortality rate of 100% and 50% was the loss of the susceptible birds. NV spoils egg quality, reduces the growth and impedes the activity of birds (Yan *et al.*, 2011). However birds expire at once in the absence of any symptom in acute cases (Ashraf and Shah, 2014). Many recent advances and discoveries have been made to surge the use of natural products, including plants as a medicament, to cure both human and animal ailments (Ruffo *et al.*, 2002; Saimo *et al.*, 2003). More than 65 plant species were used as a remedy of animal ailments in Tanzania (Minja, 1989).

Various spices including thyme, clove, oregano, mint, and cinnamon have been utilized as medicinal plant because of their antioxidant and antimicrobial properties and also as a preservative since immortal times. However, recent

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research also adds the antibacterial, antifungal, antiviral and anticarcinogenic spectra to the spice plants. Clove is exceptionally good and superlative to other spices because of its strong antioxidant and antimicrobial properties (Shan *et al.*, 2005). *Syzygium aromaticum* (*S. aromaticum*) (synonym: *Eugenia caryophyllata*) is a precious spice and commonly called as clove. It belongs to Myrtaceae family with the approximate height of 8-12 m and is indigenous to east Indonesia. The exploration, marketing and business of this worthy and precious spice upgrade the economic development of this Asiatic region up to centuries (Kamatou *et al.*, 2012).

The principal bioactive agent in the clove is eugenol. However, it also contains the crucial vegetal sources of phenolic compounds i.e. flavonoids, hydroxibenzoic acids, hydroxycinnamic acids and hydroxyphenylpropens (Neveu *et al.*, 2010). Eugenol, a compound isolated from the herbal extracts of *S. aromaticum* and from *Geum japonicum*, was identified as anti-HSV compound. Its 5µg/mL concentration was effective against herpes virus strains. The inhibitory action of eugenol is on the viral DNA synthesis by acting as selective inhibitor of the HSV-1 DNA polymerase (Kurokawa *et al.*, 1998).

Nano biotechnologies have the ability to transfigure a broad group of applications like drug delivery, diagnostics, imaging, scanning, sensing, gene knocking,

artificial implants, tissue engineering, and pest management (Elechiguerra *et al.*, 2005). The plant-mediated biosynthesis (i.e., green synthesis) of metal nanoparticles does not require the use of highly toxic chemicals, high pressure, energy, temperature and also it is inexpensive, one-stepped thus it is superior to chemical and physical methods (Goodsell, 2004). Large number of plants and fungi, with magnificent mosquitocidal properties, have recommended for effective, well organized, quick and fast extracellular synthesis of silver and gold nanoparticles (Dinesh *et al.*, 2015; Murugan *et al.*, 2015; Rajakumar and Rahuman, 2011; Rajan *et al.*, 2015; Suresh *et al.*, 2015).

The purpose of this present research is to investigate the antiviral effect of crude extracts from *S. aromaticum* against Newcastle disease virus using an in ovo assay. Poultry is the second gigantic industry of Pakistan but because of various bacterial and viral diseases its progress was affected, the most significant ailment is Newcastle disease. Newcastle disease is an ailment which is accountable for enormous deprivation in unvaccinated rural chicken inhabitant in Pakistan. This study was proposed to synthesize the green silver nanoparticles (AgNP) as a novel and productive tool against the serotype DEN-2 and its major vector *A. S. aromaticum* extract was used as reducing and stabilizing agent for synthesis of silver nanoparticles. Different biophysical methods were used to characterize AgNP including UV–VIS spectroscopy, Fourier transform infrared spectroscopy (FTIR) and sorted for size categories. Embryonated chicken eggs were used to test antiviral activity of Green-synthesized AgNP against the Newcastle Disease.

MATERIALS AND METHODS

Collection of flower buds

Clove flower buds were collected from clove tree and taxonomy identification was carried out. The identification of plant species was done by Department of Pharmacy (RLCP), Lahore, Pakistan. The herbarium number is allocated as 656/RLCP/Plant/2019. Moisture content was examined using moisture content analyzer after drying the clove buds. The buds were then pulverized into a coarse powder via grinder of 0.25 mm sieve. All the samples were then stored in desiccators after sealing them to protect them from fungal decontamination.

S. aromaticum-mediated biosynthesis of silver nanoparticles

Syzygium aromaticum buds were dried under shade at room temperature of 26°C-30°C for 2 days after washing them attentively with distilled water. An electrical blender was used to powder 20 g of buds. Now 5 g of buds powder was taken and added into 100mL of sterilized

distilled water in a 300-mL Erlenmeyer flask. The mixture was boiled for 5 min followed by filtration via Whatman filter paper no. 1. Filtrate is then stored at -4 °C and tested in no more than 5 days. The filtrate was then incubated at room temperature after treating it with aqueous 1 mM AgNO₃ solution in an Erlenmeyer flask. Silver nitrate was purchased from the Mosa-adamje-sons Scientific Co. (daejung korea). *S. aromaticum* generates stable silver nanoparticles through reduction of aqueous silver ions indicated by the dark brown solution which we have name clove extract silver nanoparticles (CESN)

Characterization of green-synthesized silver nanoparticles

The biosynthesis of AgNP was confirmed through procedure described by Murugan et al (Murugan *et al.*, 2015). The maxima absorption was obtained via UV–VIS spectroscopy at wavelength of 200–600 nm by taking 2ml of reaction mixture per sampling at regular intervals. UV-1600 Shimadzu spectrophotometer was used having 1-nm resolution. Moreover, the reaction mixture was treated to centrifugation at 15,000 rpm for 20 min. The resulting pellet was filtered through Millipore filter of 0.45µm after dissolving them in de-ionized water. A portion of this filtrate containing AgNP was used for TEM, FTIR, and XRD.

Chicken Red Blood Cells (RBCs)

The chicken blood was taken in sterilized vacutainers containing anti-coagulant which is then followed by centrifugation (Mahmood *et al.*, 2018). The RBCs were isolated from the blood at 500g for 10 minutes in the Laboratory of Department of Microbiology (RLCP). The pelleted RBCs were then washed with 0.9% normal saline three times. 25% and 1% suspension were prepared by adding 4ml and 100ml of normal saline into 1mL of packed RBCs respectively. These RBCs suspensions were used in spot and plate haemagglutination (HA) tests.

In-vitro antiviral activity

Different concentrations of clove extract silver nanoparticles (CESN) were incubating with NDV in eppendorf tubes. Spot HA test was then performed with 25% RBCs suspension along with NDV negative control (Mahmood *et al.*, 2018). The indicator of *in-vitro* antiviral activity of CESN is reduction in HA activity of virus. Similarly, 1% RBCs solution and 96-well microtitration plate was used to perform micro HA test. The detailed protocol is given below:

Spot assay

Spot assay was performed by mixing 50µl of 25% Chicken RBCs with 50 µl of NDV (pre-incubated) and various concentrations of CESN and agglutination was noticed (Vaher *et al.*, 2014). Antiviral activity of CESN is related with the lack of agglutination of RBCs.

Table 1: Spot haemagglutination test for *in-vitro* antiviral activity of CESN

S No.	Reactants	Haemagglutination property	Antiviral activity
1	NDV + (CESN)+RBCs	-ve	+ve
2	NDV + 1:2 (CESN)+RBCs	-ve	+ve
3	NDV + 1:4 (CESN)+RBCs	-ve	+ve
4	NDV + 1:8 (CESN)+RBCs	+ve	-ve
5	Positive control (NDV+ RBCs)	+ve	---
6	Negative Control (RBCs)	-ve	---

Table 2: The percentage mortality in embryonated chicken eggs post-inoculation with NDV and different concentrations of CESN

Group	Treatment	Mortality After 24 hour		Mortality After 48 hour		Mortality After 72 hour		S.D for Groups
		No. of dead embryo/ total	%	No. of dead embryo/ total	%	No. of dead embryo/ total	%	
A	Stock solution of CESN	3/10	30	7/10	70	9/10	90	± 15.28
B	1:2 dilution	4/10	40	8/10	80	9/10	90	± 15.28
C	1:4 dilution	5/10	50	8/10	80	8/10	80	± 12.02
D	1:8 dilution	4/10	40	5/10	50	7/10	70	± 13.33
E	Positive control for Virus	5/10	50	8/10	80	10/10	100	± 14.53
F	Positive control CESN	5/10	50	8/10	80	10/10	100	± 14.53
G	Negative Control	0/10	0	0/10	0	0/10	0	± 0.00
S.D for Time		± 6.85		± 11.49		± 12.88		----

*Means sharing similar letters in a row or in a column are statistically non-significant (P>0.05)

Micro haemagglutination test

Micro HA test was conducted by taking 50 µl of suspension (pre-incubated) of 4HAU of local Velogenic NDV and divergent concentrations of CESN into 96 wells microtitration plat (Nety *et al.*, 2018). After this 50 µl of 1% RBCs were added into the same wells. The 1st well and 12th well were considered as virus control and RBCs control respectively. Antiviral activity of CESN was related with the inhibition of HA activity of NDV (button formation).

Cytotoxicity assay

The maximum non-toxic dose assay was conducted by using divergent concentrations of methanolic CESN (Thabrew *et al.*, 1997). Different CESN range from buds was inoculated into embryonated chicken eggs (10 days old) through intra-allantoic route. This is then followed by eggs incubation at 37°C and candling on daily basis to determine toxicity assay i.e. to find out the maximal dose which could be non- poisonous to the chicken embryos.

In-vivo antiviral activity

In-ovo antiviral activity was assessed in 9-11 days old embryonated Chicken Eggs (ECEs) by preparing two different inoculums (Sulaiman *et al.*, 2011). The first and second inoculums were prepared by mixing 0.5ml and 0.1ml of virus with 0.5 ml and 0.9ml of different concentrations of CESN respectively.

The antiviral activity was determined through micro HA test of harvested allantoic fluids, survival of embryo and percentage of mean embryo weight

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

Preparation of inoculum (2-fold dilution of virus & CESN)

Stock solution (0.5ml) was taken and mixed with 4HAU (0.5ml) of locally isolated and characterized NDV. The mixing was done with 1:2, 1:4 and 1:8 dilutions of CESN separately in sterile eppendorf tubes followed by incubation for 60 minutes at 37°C. After which, 0.2ml of each suspension was inoculated into a lot of 10 eggs. The eggs were incubated; after sealing them; with air sac upside for three days. Eggs were inspected daily after candling them. Allantoic fluid was collected on fourth day for HA test.

Preparation of inoculum: (10-fold dilution of virus & CESN)

Stock solution (0.9mL) was taken and mixed with 4HAU (0.1mL) of NDV. The mixing was done with 1:2, 1:4 and 1:8 dilutions of CESN separately in sterile eppendorf tubes followed by incubation for 60 minutes at 37°C. After which, 0.2ml of each suspension was inoculated into a lot of 10eggs. The eggs were incubated; after sealing them; with air sac upside for three days. Eggs were inspected daily after candling them. Allantoic fluid was collected on fourth day for HA test.

STATISTICAL ANALYSIS

Data was calculated and presented as mean \pm standard deviation using SPSS 18 (SPSS Inc., USA). The least significant difference test (LSD) and ($p < 0.05$) One-way ANOVA test were employed.

RESULTS

Synthesis and characterization of silver nanoparticles

S. aromaticum generates stable silver nanoparticles; through reduction of aqueous silver ions indicated by the dark brown solution; when the AgNO_3 aqueous solution was added to the bud's extract. Extremely symmetric sole band absorption spectra of AgNP were recorded at different time intervals with the maximum absorption peak was observed at 450 nm (fig. 3) Particles are poly dispersed indicated by the broadening of the peak (Prasad and Elumalai, 2011). The peak constantly increased with reaction time, and saturated after 240 min showed full reduction of the silver nitrate. AgNP synthesized by *Syzygium aromaticum* were prominently spherical in configuration shown by TEM (fig. 2).

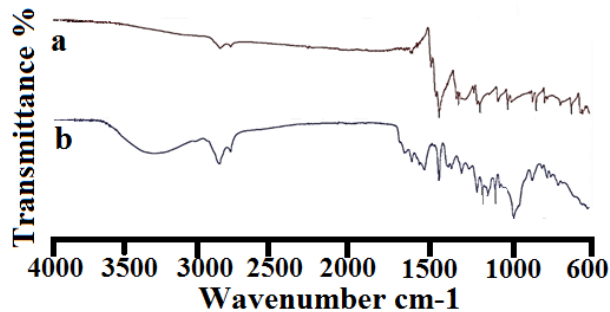


Fig. 1: FTIR study of clove buds powder extract (a) and particles (b).

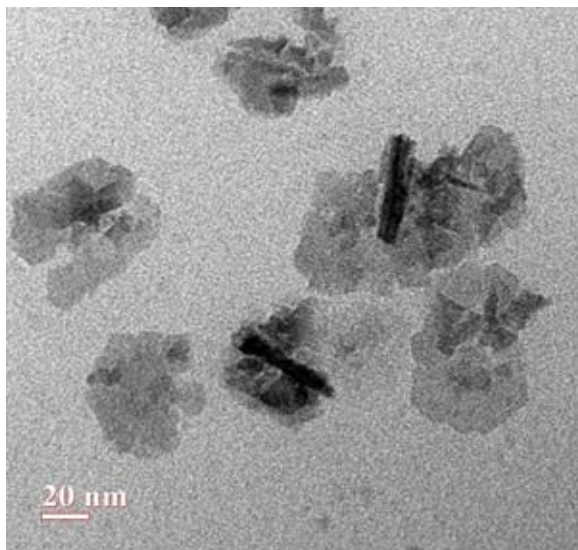


Fig. 2: TEM analysis for size determination

Characterization

FTIR analysis

FTIR analysis of silver nanoparticle established the characteristics of the plant extract as a reducing and capping agent and also shows the existence of some functional groups. Clove buds powder extract (a) showed instinct absorption bands at 3423.15 and 3420.77 cm^{-1} which indicated the presence of phenols and alcohols with free OH group. This band is superimposed by NH stretching peak. The bands at 2928.45 and 2928.44 cm^{-1} represent the presence of alkanes in lipids. The region of 2359.03 cm^{-1} indicates the presence of symmetric stretching of COO^- just in the biosynthesized solution (Banerjee *et al.*, 2014). In fig. 1(b) part belongs to the CESN in which free carboxylate groups presented in proteins can bind to the metal nanoparticles and make them stable. Only minor changes in the position of absorption bands between FTIR spectrum of the clove buds powder and biosynthesized CESN were seen. The involvement of clove buds powder extract in biosynthesizing of nanoparticles was confirmed with shifting of peaks. Thus, the extract compounds including OH and CO groups have a vital role in reducing and stabilization of NP.

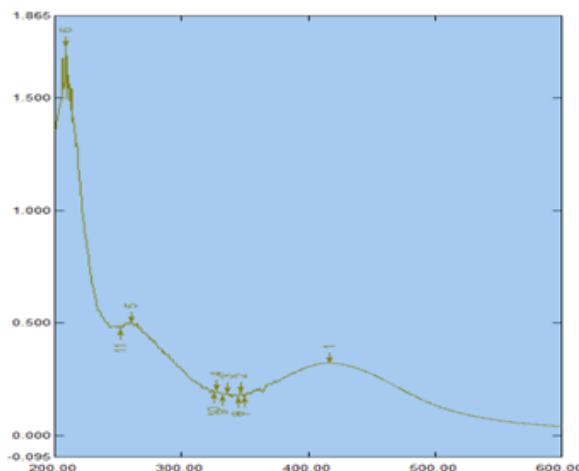


Fig. 3: UV-VIS absorption spectra of CESN

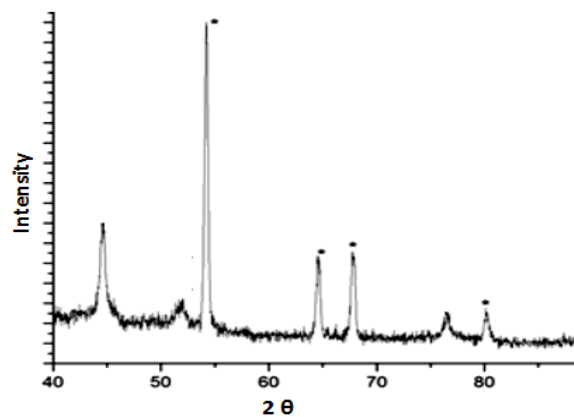


Fig. 4: XRD peaks of CESN

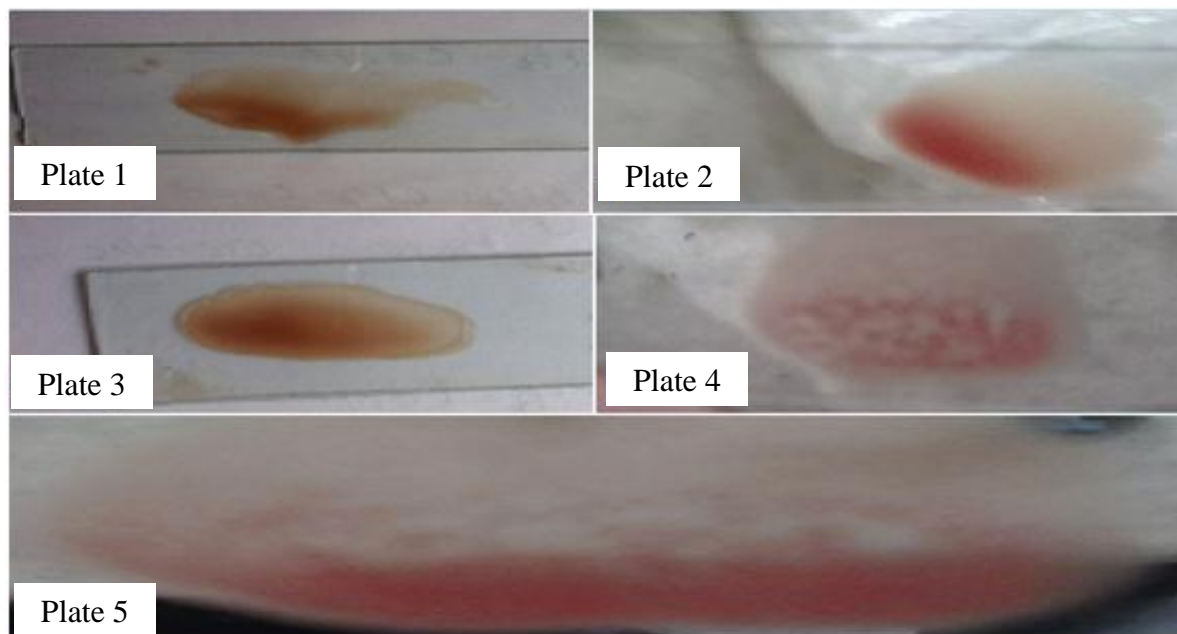


Fig. 5: Plate 1: Spot HA test with stock solution of CESN, Plate 2: Spot HA Test with 1:2 dilution of CESN, Plate 3: Spot HA test with 1:4 dilution of CESN, Plate 4: Spot HA Test with 1:8 dilution of CESN, Plate 5: Spot HA test with NDV alone (Positive Control).

TEM analysis

The size, shape and morphology of nanoparticles were identified by Transmission electron microscopy (TEM). It discloses that the silver nanoparticles are uniformly distributed and primarily unanimously spherical in nature but some were irregular shape as shown in fig. 2.

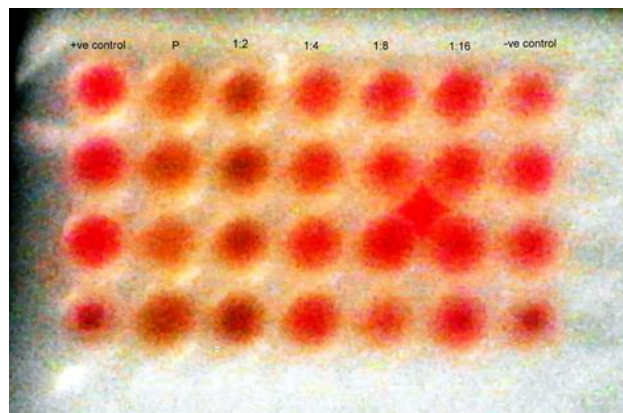


Fig. 6: Micro haemagglutination test of NDV treated with CESN.

UV-Vis analysis

UV-Vis absorption spectrum of AgNP is shown in fig. 3. Broad bell-shaped spectrum curve was obtained from UV-VIS analysis. Various metabolites from plant extract introduced to solution make the plasmon band broad because they may be read in this spectrophotometric range, too. Surface plasmon resonance (SPR) of silver occurs at 450 nm. This peak increased with time up to 360 min. According to Mie theory, spherical nanoparticles

show only a single SPR band. The number of peaks increases by increasing diversity of particles shapes. Then, it can be concluded that biosynthesized AgNP are unanimously spherical in nature.

X-ray diffraction analysis

XRD pattern revealed distinct peaks at 2θ values, which can be attributed to 111, 200, 220 and 311 crystalline planes of AgNP (fig. 4). These peaks are associated with the facecentered cubic lattice. Other peaks at 2θ values in AgNP pattern can be ascribed to the residues of the organic content of the clove powder extract. These peaks reveal the crystallization of some plant metabolite moieties on the surface of the AgNP, which is in agreement with Shanmuganathan et al.'s results. This result is in accordance with XRD analysis of Oves *et al.*

Spot haemagglutination Test

Spot assay showed the antiviral activity with the lower dilution only. The stock solution of CESN and 1:2 dilutions inhibits the visible clusters / agglomerates of chicken RBCs. The results of spot HA test shown in table 1. The stock solution, 1:2 dilution and 1:4 dilution of CESN exhibited antiviral activity but it showed toxicity to RBCs by changing its color. However higher dilutions of clove buds extract neither inhibit clusters of RBCs by NDV nor the change in color. These results are shown in Plate 1 through Plate 5.

Micro haemagglutination test

The outcome of Micro HA test was in distinguishable to that of spot HA test. Microtitration plates were used for

the test that contains NDV. Wells were treated with 1:2, 1:4, 1:8 and 1:16 of CESN. 1:2 well showed antiviral activity by inhibited the HA property of virus however at the same time. Higher dilutions (1:4, 1:8 and 1:16) did not showed any antiviral activity and did not inhibit the HA property of virus. In first three wells, there was a remarkable change in the hue of RBCs suspension from glowing red to pale/serum neither like hue with neither clumping nor button formation.

Cytotoxicity assay

For the maximum non-toxic dose assay different concentrations of CESN were injected into ten days old embryonated chicken eggs through intra allantoic route. This is then followed by eggs incubation at 37°C and candling on daily basis to determine toxicity assay i.e. to find out the maximal dose which could be non-poisonous to the chicken embryos. The test was performed thrice times and average result were taken. It was depicted in result that stock solution of CESN, 1:2 and 1:4 dilutions showed cytotoxicity to embryonated chicken eggs *in-vitro* while with higher dilutions no toxicity was noted.

DISCUSSION

Now a days medicinal phytochemicals have captivated concentration because of their beneficial effects on the health, production and behavior of the birds by boosting immune system of the body and by elevating antimicrobial potential (Alloui *et al.*, 2014). NDV has been graded in list A by the World Organization for Animal Health as it is amongst the most significant diseases of poultry birds, because it severely influences the worldwide poultry production (Fernandas *et al.*, 2014). Specific plant extract can inhibit the NDV activity. Waihenya *et al.* (Waihenya *et al.*, 2002) checked the effect of Aloe secundiflora, Salmonella gallinarum and other bacterial varieties in chickens against NDV but no prophylactic or beneficial effects were reported. Similar studies were performed using crude extracts of Capsicum flutescens, Citrumlimon and Oputia vulgar but again no effect against ND virus in chickens was observed (Bakari *et al.*, 2012). Negative results were also obtained with stems of Euphorbia candelabrum (Euphorbiaceae), fruits of Capsicum annum (Solanaceae) and Ibozamultiflora (Liliaceae) (Minja, 1989).

Present study was conducted to evaluate the antiviral activity of *S. aromaticum* against ND virus. 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 (fig. 5), it was observed that stock solution of CESN and 1:2 dilutions inhibits the visible clusters / agglomerates of chicken RBCs. The results of spot HA test shown in table 1. The stock solution, 1:2 dilution and 1:4 dilution of CESN exhibited antiviral activity but it showed toxicity to RBCs by changing its color.

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

This study focused at evaluating the antiviral effects of clove buds extract modified silver nanoparticles against NDV infection, *in vitro* and *In-vivo* at 1:8 concentrations that are not deleterious to the host cells. *S. aromaticum* modified AgNP provided the increased effectiveness as inhibitory agent against virus as depicted by the results.

CONCLUSION

The current article showed that the buds extract of extensively accessible medicinal plant *S. aromaticum* could be utilized for biosynthesis of silver nanoparticles. This single step method is very appropriate, suitable, economic, nature-friendly and thus is an optimistic and suitable substitute of conventional way of preparation. In conclusion, CESN showed significant antiviral activity at higher concentrations during both *in-vitro* and *in-vivo* trials. Reduction in concentration is directly proportional to the reduction in antiviral activity and cytotoxicity CESN have shown promising efficacy as an antiviral agent and can be used as an effective antiviral alternate by minimizing its cytotoxic effects.

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