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Journal of Drug Delivery and Therapeutics

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Research Article

In-vivo antiviral potential of crude extracts derived from *Tribulus terrestris* against newcastle disease virus

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ABSTRACT

Viral problems have been in focused of the scientists due to their high metabolic rate, drug resistance and unique nature of pathological mechanism. The failure of novel synthetic allopathic antiviral drugs propels the scientists to investigate other sources of alternative antiviral agents. Thin layer chromatography (TLC) and spectrophotometry were conducted by using standard methods of phytochemical analysis of bioactive components. The methanolic extracts of *Tribulus terrestris* showed higher phytochemical phenols followed by tannins, alkaloids, carotenoid, saponins quantified by spectrophotometer assay. The current study was done to evaluate the in vivo antiviral potential of crude extracts of medicinal plant by means of NDV Haemagglutination (HA) titer in vivo vero cell line culture. Furthermore, different doses of crude extract such as 20µl/ml, 40µl/ml, 60µlml and 80µl/ml were interacted with Lasota strain of the NDV (EID_{50}= 1×10^5, HAU= I0^5) in 90% saturated vero cell line with constant supply of CO_2 at 37°C. It is evaluated that the prevention dose (80µl/ml) of *Tribulus terrestris* (Pre-treatment) against Newcastle disease virus on vero cell line (2.5±1.0) just before 24 hour of infection is declared as optimum effective time period to counter these agents as compared to Co-treatment (80ul/ml) (3.0±1.15) and Post treatment (80µl/ml) (3.50±1.00). It is suggested from the results of current study that *T. terristris* showed enormous anti Newcastle disease virus effect in vero cell line adapted virus particularly when used as preventive antiviral therapy at the dose rate not less than 80ul/ml just before onset of disease.

Keywords: Thin layer chromatography (TLC), Antiviral activity, *Tribulus terrestris,* Haemagglutination (HA) titer

Article Info:

Cite this article as:

Malik A, Mehmood MD, Anwar H, Noreen S, Sultan U, *In-vivo* antiviral potential of crude extracts derived from *Tribulus terrestris* against newcastle disease virus, Journal of Drug Delivery and Therapeutics. 2018; 8(6):149-154
DOI: http://dx.doi.org/10.22270/jddt.v8i6.2114

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INTRODUCTION

Poultry is the second largest industry of the Pakistan has been providing cheap and quality protein to the fast growing consumers. The poultry industry of Pakistan is continuously threatened by variety of infectious and non-infectious agents (Gueye *et al*., 2002)[1]. However, infectious agents such as bacteria, viruses and fungi have been reported as dominant factor posing damage to the industry. The commercial birds are consistently being immunized against viral problems such as influenza, Newcastle disease virus, infectious bronchitis virus, hydro pericardium syndrome disease virus and infectious bronchitis virus.

The heavy mortality has been observed even in immunized commercial broiler flocks which may be due to genetic recombination of pathogenic gene. The resulting change in the gene may cause ineffective neutralization with already induced antibodies. Secondly, the process generates resistance to the available synthetic antiviral drugs. So, there is dire need to search other alternative measures for the prevention of high mortality and morbidity in birds particularly caused by NDV[2] (Omer *et al.,* 2014). The causative agent is a single stranded, enveloped, non-segmented RNA virus belongs to paramyxoviridae family[3] (Zamarin *et al.,* 2009). Two viral glycoprotein's Haemagglutinin neuraminidase protein (HN) and fusion protein (F) are responsible for the NDV infection. (Samal *et al.,* 2000). NDV strains are classified into three major pathotype: lentogenic, mesogenic and velogenic. Lentogenic strain is not pathogenic in adult chicken and used as live vaccines in poultry worldwide. Mesogenic strains cause respiratory disease in birds, while the most pathogenic strains of NDV are lentogenic that causes the high mortality rate in birds. Nine paramyxovirus serotypes (APMV-1) to (APMV-2) has been reported[5] (Arfin *et al.,* 2010).

There are several difficulties in developing antiviral agents. Majority of the approved antiviral drugs possess adverse effects and have also developed viral resistance in long-term therapy[5] (Mishra, 2013). Natural products provide important sources in research of new antimicrobials for the inhibition of microorganism's growth. Many medicinal plants produce phytochemicals that are active against bacteria and viruses. These chemicals have the ability to stop the growth of multi drug resistance organisms[6] (Aziz *et al.,* 2017).

Herbal medicines are one of the most rational medicines in the world especially in ruler areas. Plants contain certain bioactive components which inhibit the growth of microorganisms. Photochemical like alkaloids, flavonoids, saponins and tannins are the novel antiviral agents[7] (Raza *et al.,* 2016). Among all photochemical, saponins are recorded beneficial for research to rule out the alternatives for controlling the diseases in poultry[8] (Vicente *et al.,* 2007). *Tribulus terresteris* (*T. terrestris)* is medicinal plant of family zygophyllaceae which is used by many pharmaceutical industries as an effective source of medication (Deshwal *et al.,* 2014). The fruit of *Tribulus terrestris* is beneficial for skin diseases, antibacterial anti inflammation, antiviral, dyspepsia, cough, asthma, cardiopathy and hypertension[11] (Sivarajan and Balachandran 1994). In *T. terrestris* steroidal saponin present which are considered the main factor responsible for the pharmacological action. The composition of the saponins is different in different geographical regions[12] (Dinchev *et al.,* 2008). The current study was designed to develop the standard protocol for the crude extraction of *Tribulus terrestris* and identification of bioactive component by Thin layer Chromatography (TLC). Furthermore, probable anti-NDV mechanism of action at certain dosage of crude extract has been determined in vero cell line culture under controlled conditions.

METHODOLOGY

Source of plant:

500 grams processed fruits powder of *Tribulus terrestris was* purchased from the reputed herbal supplier (Atari pansar store) Akberi mandi, Lahore C/O reputed herbal laboratory Hamdard (WAQF) Pakistan.

Washing and Drying:

The powders were dissolved in enough sterile distilled water (pH-7.2) and got filtered through sterile muslin cloth. The process repeated three times and was kept in hot air oven at 100°C for 30 minutes. The moisture free powder was stored in cool dry place till further processing.

Preparation of methanol extract:

100g of fine ground powder of *Tribulus terrestris* was mixed with 400ml of each methanol and ethanol in separate pyrex flasks. It was shacked gently until clear suspension was obtained and kept in the lab for 4 days. After four days the suspension was spread on the glass petri plates (9mm) for the evaporation of oils. The solution was filtered through 0.45µm filter paper (Whatmann-Germany) and centrifuge at 12000 RPM for 9 min. The supernatant was lyophilized at -45°C for about 8 hours.

Preparation of TLC plates:

Commercially available TLC plates were cut down to smaller strips according to the TLC chamber with the dimensions of 1"x4".With the help of pipette 2ml of following solvents were added in the TLC chambers.

- TLC chamber 1: Ethyl Acetate + Hexane 1ml: 5ml
- TLC chamber 2: Ethyl Acetate: n-hexane in the ratio of 1:1ml
- TLC chamber 3: Ethyl Acetate (pure)
- TLC chamber 4: Chloroform (pure) will be used.
- TLC chamber 5: Chloroform: methane in ratio of 30:1ml
- TLC chamber 6: Chloroform: methane in the ratio of 15:1ml
- TLC chamber 7 and 8 Chloroform: methanol in the ratio of 7:1ml and Chloroform and methanol in the ratio of 3:1ml respectively.

Loading and labeling of samples:

Each of the strips was marked in the bottom and spotted with respective methanolic crude extract and each strip was shifted to its respective chamber containing solvent as below.

Incubation and observations:

The sample loaded strips were incubated for 15 mints with their respective solvent and the movement of segregated band was observed under UV–light. Each of the TLC strip was sprayed with vanillin and sulphuric acid in the ratio of 1:4ml. The plates were dried in the hot air oven.

Quantification:

Total phytochemicals and steroidal saponins of the crude extract were analyzed by spectrophotometric method (REF).

Dose adjustment:

The solvent free crude extract was freeze dried using lypholizer. Furthermore, each one milli gram of freeze dried crude extract was dissolved into sterile normal saline solution to prepare different dosage concentration. Subsequently, different concentrations such as 20µg/ml, 40, 60, and 80 were subjected for the antimicrobial activity against Newcastle disease virus TCID50 $1x10^6$/ml. Following concentrations of crude extract containing different amount of active ingredients were evaluated for its potential antiviral activity.

Source of virus:

The characterized inactivated Newcastle disease virus (N/Broiler-Chicken/OP/OLV/14 NDV) TCID50-$10^{5.6}$ /ml, HA 264 unit/ml virus was obtained from Ottoman Pharma (Immuno Division) licensed veterinary vaccine manufacturing company located at 10-km Raiwind road, Lahore.

Source of cell line:

The characterized Vero cell line was obtained from cell and tissue culture section of center for research in Molecular Medicine the University of Lahore, Pakistan.

Preparation of DMEM:

Dulbecco's Modified Eagle Medium (DMEM) was prepared according to the instructions of manufacturer (Glassgo-UK).

Reactivation of Vero cell line:

The primary culture of cells were trypsinized (Gibco) and transferred to T-25 flasks containing 5 ml of DMEM. Then flask was incubated at 37°C for 48 hour with 5% CO_2 in atmosphere and humidified incubator.

Sub-culturing of cell lines:

After 48 hour, the flask containing reactivated cell lines was sub-cultured to 6 well plates using 0.25% trypsin solution supplemented with 0.5mM EDTA. Before virus inoculation or results quantification, the monolayers were washed thoroughly twice with phosphate buffered-saline (PBS, pH 7.4 at room temperature).

Dilution of extract:

Each extract of the respective plant was diluted at the ratio of 1:100 with help of sterile normal saline. It was shacked gently to get complete dissolution and kept it for 12 hours to settle down any larger particle in the suspension. The sediment was decanted and the clear supernatant used in the process.

Dose-based anti-NDV activity:

Doses such as 20µl, 40µl, 60µl and 80µl were added in 6 well tissue culture plates containing 1x 10^5 cell/ well. The well 1, 2, 3, 4 and 5 were marked, inoculated with 20µl, 40µl, 60µl and 80µl respectively and incubated for 24 hours at 37°C in 5% Co_2 incubator and the results were recorded. Whereas, 6th well was kept as a negative control.

Mechanism- based anti-NDV activity:

Different doses of each plant extract were inoculated separately in 6 well tissue culture plates at different time interval. All the wells contain uniform vero cells growth of 1x 10^5 well 1st and 2nd were treated with 80ul of each extract before one hour of infection, whereas 3rd and 4th were simultaneously treated followed by 5th and 6th well where infection was given one hour before the treatment. All well in the plate B was kept as non-medicated control. The process was repeated 4 times.

Evaluation:

The virus was titrated by Haemagglutination assay (HA) following the procedure advocated by Allan *et al.*, 1978. Two-fold serial dilutions of the virus were made in normal saline beginning with 1:2 dilutions in first well through 12th well. A total of 0.5 ml red blood cells (RBCs) suspension (1%) was added in all the wells. The plate was gently mixed by tapping before incubating at room temperature for 45 minutes. HA was determined by observing the presence or absence of bottom shaped streaming of RBCs. The titration was read to the highest dilution of the virus giving complete HA (no streaming) which represented 1HA unit (HAU) (Allan *et al.*, 1978).

Statically analysis:

The data obtained was analyzed through mean Standard Derivation (SD) and subsequently by ANOVA.

RESULTS

Vertical TLC chromatogram was developed which was visualized under UV light (350nm). Freeze dried ethanolic and methanolic TLC analyzed products of *Tribulus terrestris* were observed as green color crystals. The distance travelled by the spot was 1.7 cm and that distance travelled by the mobile phase was 5 cm, so the retardation factor (R_f value) = 1.7/5.0=0.34 cm. (R_F =distance travelled by the solute /distance travelled by the mobile phase).Whereas, Vanillin sulphuric acid (2 % solution of vanillin in phosphoric acid 1:4 detected the steroidal saponin by grey blue color band.

Chromatogram under UV-light:

Spectrophotometery analysis reveals the presence of total and steroidal saponin as the absorbance was R_1= 0.145, R2= 0.152, R3= 0.148 and R1 =1.780, R2= 1.779, R3= 1.777 respectively. The value of total saponin and steroidal saponin was calculated by the following equations

Y= 0.0005X - 0.0052, Y= 0.107x+0.138 (Where y =absorbance, x =concentration). The value of total saponin was 286.6 µg /g of dry extract and steroidal saponin was 15.2 µg /g of dry extract.

TLC analysis revealed the presence of phenolic contents 14.33±2.16, Tannins 16.29±3.99, alkaloids33.26±4.77, flavonoids 126.25±10.26, carotenoids 19.59±3.29, total saponins286.6±3.16 and steroidal saponins15.32±2.16 (Table-1).

Table 1: Total phenololic, tannin, and alkaloid flavonoid and saponins contents in the ethanolic extracts of *Tribulus terrestris*

Tribulus terrestris	**Phenols** µg of GAE/g of extract (R=10**)**	**Tannins** µg of GAE/g of extract (R=10)	**Alkaloids** µg of GAE/g of extract (R=10**)**	**Flavonoids** µg of QE/g of extract (R=10)	**Carotenoids** µg of GAE/g of extract (R=10)	**Saponins (Total)** µg of D/g of dry extract (R=10)	**Saponins (Steroidal)** µg of D/g of dry extract (R=10)
	14.33±2.16	16.29±3.99	33.26±4.77	126.25±10.26	19.59±3.29	286.6±3.16	15.32±2.16

Note: Each value is the average of three analyses (Mean) ± standard deviation (SD), where "GAE" is Gallic acid equivalents, "Q" is Quercetin equivalents and "D" Diosgenin is equivalents **Standard curves**

1-Phenols (Y=0.0037x+0.031, R^2=0.9497), **2-Tannins** (Y=0.0287x-0.7049, R^2=0.9164) **3-Alkaloids** (Y=0.0112x=0.1755, R^2=0.9472), **4-Flavonoids** (Y=0.0034x-0.0598, R^2=0.9208) **5- Carotenoids** (Y=0.01x-0.2397, R^2=0.9409), **6-Saponins (Total)** (Y=0.107x+0.138, R^2=0.923) **7-Saponins (Steroidal)** (Y=0.0005x+0.0052, R^2 = 0.941)

Different doses of the plant extract such as 20µg/ml, 40µg/ml, 60µg/ml and 80µg/ml were interacted with Lasota strain of Newcastle disease virus (NDV) at different time interval in 90% saturated vero cell line with constant supply of 5% CO_2 at 37°C. It is evaluated that one hour Prior infection, co-treatment and post infection of *Tribulus terrestris* (80ug/ml) to NDV infected vero cell line showed 2.5±1.0, 3.0±1.15 and 3.50±1.0 mean standard deviation (M±SD) respectively of HAU (Fig. 2). Similarly, untreated control group inoculated with the same dose of NDV with same conditions (M±SD) 320.0±128.0 of HAU titer.

DISCUSSION

Synthetic drugs emerged as immediate and successful solution for the treatment of viral problems throughout the world but natural sources can never be undermined as most of the established drug molecules trace their route to natural sources directly or indirectly. Since time

immemorial Saponins are use in various parts of the world for short treatment of diseases (Ganwal A 2010)[13]. In order to make them available for treatment of various viral infections their extraction is vital step in the preparation of antiviral medicine. The conventional extraction methods are maceration, soxhlet and reflex extraction by using different extraction solvents like methanol, ethanol, water, chloroform, ethyl acetate and n hexane etc (Singh *et al* ., 2015)[14].

Thin layer chromatograophy (TLC) has been used in the current study to analyze the different components particularly saponins in the methanolic and ethanolic crude extracts of *T. terrestris.* Moghimipour *et al.,* 2015 support the results of current study in which saponins were isolated and identified by TLC using different solvents in different ratio for the detection of saponins[15]. Our results pertaining to total saponins and steroidal saponins in *Tribulus terrestris* was quantified by spectrophotometric assay. Spectrophotometry analysis revealed the presence of total saponin was 286.6µg D/g of dry extract and steroidal saponin was 15.2µg D/g of dry extract (Table-1). Our observations do agree with Singh *et al*., 2015 who declare that total saponins and steroidal saponins in crude extract of *Abutilon indicum* leaves were 296±9mg DE/gm and 7±0.2mg DE/gm respectively.

The methanolic quantitative analysis of *T. terrestris* showed phenols = 14.33±2.16µg of GAE/g of extract, Tannins =16.29±3.99µg of GAE/g of extract, Alkaloids =33.26±4.77µg of GAE/g of extract, Carotenoid = 19.59±3.29µg of GAE/g of extract, Saponins (Total) = 286.6±3.16µg of D/g of dry extract, Saponins (Steroidal) = 15.32±2.16µg of D/g of dry extract quantified by spectrophotometer assay as shown in (Table-1). Widyawati *et al*., 2014 used similar technique and solvent to determine difference of solvent polarity. According to his observations major photochemical in *Pluchea* leaves was polarity properties extracted by methanol and ethanol including saponin, flavonoids, alkaloids, tannins and reducing sugars. Methanolic extracts had the highest total flavonids (911.9mg GAE/g samples db) and total phenols (1185.2mg GAE /g samples db). Yield obtained from methanol solvent extraction of *Pluchea* leaves were 38.07 ± 2.08 % while ethanol extract had given yield % of 31.09 ±1.75.

Anti-viral agents have been consistently focused by the scientists because of unique nature of viral replication and evolution of new diseases in both poultry and human population. *T. terrestris* various plants extracts for their reported antiviral activity in association with mechanism of action and least effective dosage in vero cell line adapted Newcastle disease virus. *T. terrestris* extract did not show any cytopathic effect in vero cell line up to 100µl/ml ($p<0.05$). Our results are also supported by (Abudayyak *et al.,* 2015) who mentioned that 160µg/mL of *Tribuls terrestris* methanol extract up to IC_{50} value had not shown any cytotoxic effects on NRK-52E cell line.

It confirms the efficiency of *Tribulus terrestris* against Newcastle disease virus showing 80µg/ml of purified freeze dried methanolic extract has significantly ($p<0.05$) (2.5±1.0) higher anti-NDV response as compare 60µg/ml (5.0±2.0), 40µg/ml (12.0±4.6), 20µg/ml (20.0±8.0) in vero cell line. (Figure-1). Yazdi *et al*., 2014 conducted similar studies in which1g/kg of *Tribulus terrestris* offered in feed to broiler chicken as part of basal diet resulting into development of better antiviral response as compared to the control group[17].

Many pharmacological activities have been reported about saponins such as antibiotic, antifungal, antiviral, hepatoprotective anti-inflammatory and anti-ulcer (Oakenfull and Fenwick 1981; Chao *et al*., 1998). According to our study saponins show no cytotoxicity to HEPG-2 cells up to 80ul/ml, our result was supported by (Pu *et al*., 2015) declaring that saponins had no cytotoxicity on MDCK cells at the concentration of 50µg/ml[20]. The study revealed significant ($p<0.005$) anti-NDV effect in pre–treatment assay (80ul/ml) (5.0±2.0) in comparison to Co-treatment (80µl/ml) (8.0±0.0) and post treatment (80µl/ml) (24.0±9.2). The result are in agreement with Johnson, 2013 who highlighted that Pre-treatment assay showed 60% reduction in number of plagues when infected with Rotavirus, while reovirus show decrease in viral production up to 74% after 6hr pretreatment with saponin.

It is observed from the results obtained in the current study that *Tribulus terristris* has showed enormous anti Newcastle disease virus effect in vero cell line adapted virus particularly when used as preventive antiviral therapy at the dose rate not less than 80ul/ml just before onset of disease.

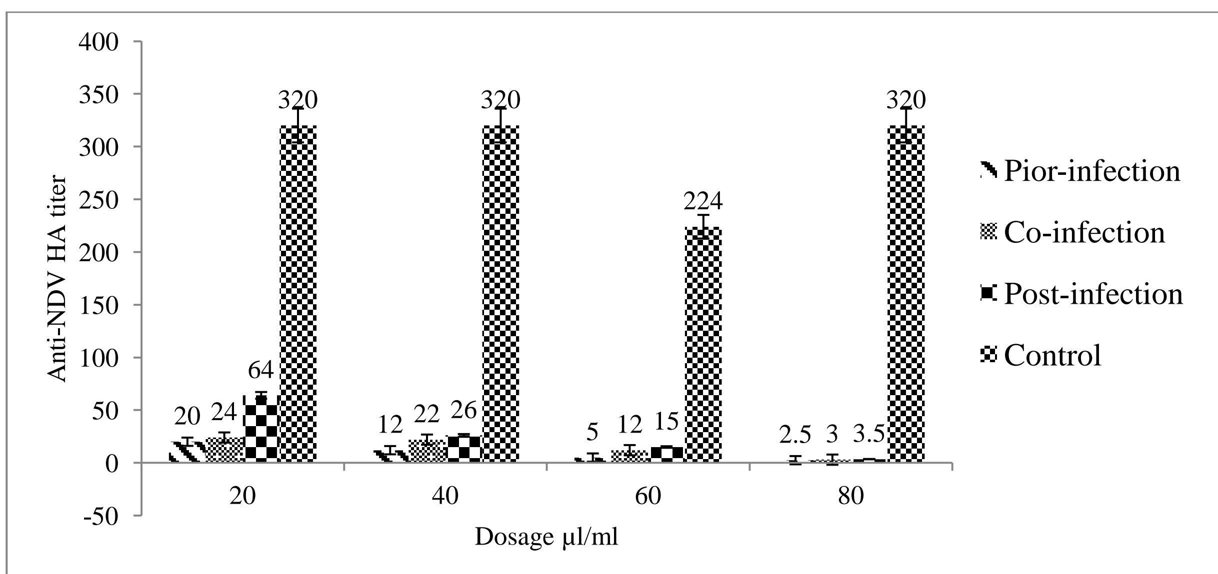

Figure 1: Dosage associated mechanism of action of *T. terrestris* on NDV

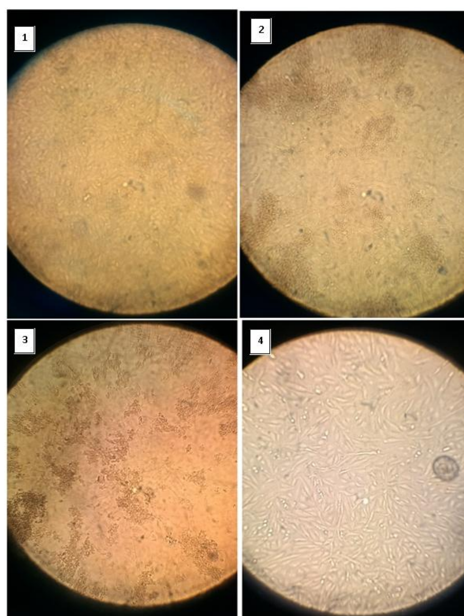

Figure 3: Microscopic analyses of cell line. 1: Pre-Treatment Assay, 2: Simultaneous Treatment, 3: Post- Treatment Assay, 4: Control.

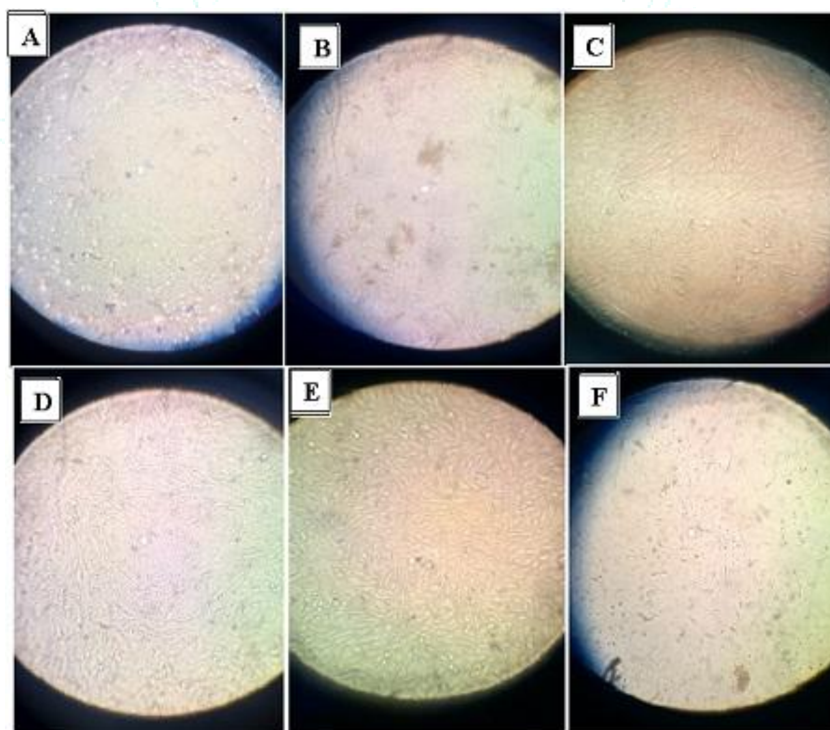

Figure 4: Microscopic analyses of anti-NDV activity on vero cell line. A: 20 µl, B 40 µl, C: 60 µl, D: 80 µl, E: positive control (media), F: negative control (virus)

CONCLUSION

It is concluded that high content of crude *T. terrestris* recovered by using ethanol as solvent and its components could be analyzed through thin layer chromatography. Potential antiviral agent saponin was documented as the major bioactive component in crude extract, whereas 80µl/ml of freez dried *T. terrestris* crude extract showed anti-NDV activity on vero cell line without any ill effect ($p<0.05$). Moreover, *T. terrestris* alcoholic crude extracts would be highly helpful when used as precautionary measures in order to control NDV outbreaks.

ACKNOWLEDGEMENT

The authors of the paper are highly grateful to Dr. Muhammad Danish Mehmood the director of Ottoman Pharma (Immuno Division) for providing financial support, isolate of Newcastle disease virus and laboratory facility to execute the research in the best interest of the poultry industry.

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