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In Vivo Anti-Viral Effect of *Melaleuca alternifolia* (Tea Tree Oil) and *Olea europaea* (Olive Leaf Extract) on Vero Cell Adapted Avian Influenza 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 current study was under taken to evaluate the in vivo antiviral potential of purified extracts of medicinal plants by means of AIV Haemagglutination (HA) titer in-vivo vero cell line culture. Different doses were interacted with H7N3 ($TCID_{50}$= $1\times10^{5.6}$, HA= 264 HA unit/ml) and H9N2 ($TCID_{50}$= $1\times10^{6.2}$, HA= 264 HA unit/ml) strain of the AIV in 90% saturated vero cell line It is evaluated that 10ul of *Melaleuca alternifolia* (TTO) and *Olea europaea* (OLE) pre-treated vero cell line showed significant anti influenza H7N3 (0.5+43) and H9N2 (1.0+45) mean HA titer as compare to non-medicated control group (320.0±128.0) inoculated with the same dose of AIV provided with same conditions. Thus, maximum dose (10µl) of *Melaleuca alternifolia* showed significantly similar antiviral mean HA titer (0.5±1.0) ($p<0.05$) response on vero cell infected H7N3-AIV when treated 24 hour, but significantly higher than *Olea europaea* (3.0±1.1) in terms of mean HA titer. However, either of the selected plants did not show any cell toxicity by means of cytopathic effect (CPE) and Haemagglutination activity alone or synergistically even at 100µg/ml. Results based on the current study would suggest the use of *Melaleuca alternifolia* and *Olea europaea* in poultry as prevention may help to control AIV outbreaks.

INTRODUCTION

Antimicrobial agents are those chemicals which kill or stop the growth of micro-organism. It can be grouped into one class according to their mechanism of action. Whereas Antiviral drugs are those compound that prevent increase and propagation of a virus without causing any significant damage to the host and hence, can be used to treat infections. Most of the designed antiviral drugs are being used to limit avian influenza and new castle disease virus (Ding *et al*., 2017).

The influenza virus is a member of the Orthomyxoviridae family, a group of enveloped viruses that possess negative-sense, segmented, and single-stranded RNA genomes. The virus causes large-scale morbidity and mortality in variety of animals that includes human, birds, horses, pigs and sea mammals, causing significant economic loss worldwide and in Pakistan (Palese and Shaw, 2007). AIV Subtypes particularly H5N1, H7N2, H9N2 were first reported over 10 years in Asia (Sims *et al.*, 2005), whereas the 2009 pandemic Influenza A H1N1 was first reported in April of 2009 in the USA (MMWR, 2009b). The most recent AIV subtype H7N9 outbreak has been reported in China (Gao *et al.*, 2013; Liu *et al.*, 2013).

The surface of AIV comprises two major glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Due to the antigenic drift of these two proteins, AIV is classified into 16 haemagglutinin subtypes (H1-H16) and 9 neuraminidase subtypes (N1-N9). The possible combinations of influenza A virus with both HA and NA form 144 sub-types and so far 103 have been found in wild birds. The complete AIV genome is 13,588 nucleotides long and consists of 8 segments of single stranded, negative sense ribonucleic acid (RNA): haemagglutinin (HA) gene; neuraminidase (NA) gene; matrix (M) gene; nonstructural gene; nucleoprotein (NP) gene; and three polymerases: PA, PB1 and PB2 genes (Alexander., 2007).

Tea tree oil (TTO) is a popular ingredient used in vast array of cosmetic and medicinal products around the world. The antimicrobial activity of TTO, the essential oil that is distilled from the Australian plant *Melaleuca alternifolia*, have been proven by a number of Australian (Southwell *et al.*, 1993; Carson *et al.*, 1996; Hammer *et al.*, 1999) and international (Ånséhn, 1990; Banes *et al.*, 2001) studies. In order to demonstrate the TTO anti-viral activity against Avian Influenza viruses, this study is designed to verify and evaluate its use in treating viral infections.

It is known that the *Olea Europaea* fruit, its leaves and its oil have a rich history of medicinal and nutritional uses (Soni *et al.*, 2006). Oleuropeosits (oleuropein), flavonols, flavones and substituted phenols (hydroxytyrosol, tyrosol) are some phenolic compounds presesnt in the olive leaf extract (Benavente *et al.*, 2000). It has been stated by various researchers that the olive leaf extract has an antimicrobial activity due to the presence of its high phenolic content (Markin *et al*., 2003; Sudjana *et al.*, 2009).

Olive Leaf Extract also inhibits HIV-1 replication as assayed by p24 expression in infected H9 cells (Lee-Huang *et al.*, 2003). In the light of aforementioned limitation regarding unsuccessful story of antiviral treatments scientists have been consistently focusing some other alternatives. The primary objective is to discover some naturally occurring antiviral agents and it was observed that plants offer us a diversity of therapeutic metabolites which have potential to inhibit viral replication by inhibition of virus penetration into the host cell, regulating viral adsorption, binding to cell receptors and by competing for pathways of activation of intracellular signals.

In the present study, TTO and Olive oil were evaluated for its potential therapeutic activity interacting different concentration of medicinal plant extracts with known amount of specific virus quantity under controlled conditions.

METHODOLOGY

Source of Plant Extract:

Purified extract of *Olea europaea* leaves and *Melaleuca alternifolia* was purchased online from Shopping Bag and Daraz respectively (Fig. 1).

Source of Virus:

The characterized inactivated Avian influenza H_7N_3 (A/Breader-Chicken/OP/OVG/17) $TCID_{50}$-$10^{8.1}$ /ml, HA-264 HA unit/ml and H_9N_2 (A/Breader-Chicken/OP/OVI/12) $TCID_{50}$-$10^{8.6}$/ml, HA-264 HA unit/ml virus (Mehmood et al., 2016) were obtained from Ottoman Pharma (Immuno Division) licensed veterinary vaccine manufacturing company located at 10-km Raiwind road, Lahore, Pakistan.

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 0.5 mM EDTA. Before adding the virus or when quantifying the results, the monolayers were washed thoroughly twice with phosphate buffered-saline (PBS, pH 7.4 at room temperature).

Before virus inoculation or results quantification, the monolayers were washed thoroughly twice with phosphate buffered-saline.

Dilution of Extract:

The solution was diluted according to the instructions of manufacturer mentioned on labeled container as below:

1 full dropper - 30 drops

30 drops - 250 mg

1 drop - 250/30 - 8 mg

It was shaken 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-influenza activity of extracts:

Doses such as 2 µl, 4 µl, 6 µl, 8 µl and 10 µl 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 2 µl, 4 µl, 6 µl, 8 µl and 10 µl respectively and incubated for 24 hours at 37°C in 5% Co_2 incubator and the results were recorded. Whereas, 6^{th} well was kept as a negative control.

Mechanism based anti-influenza activity of extracts:

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 1^{st} and 2^{nd} were treated with 10ul of TTO before one hour of infection, whereas 3^{rd} and 4^{th} were simultaneously treated followed by 5^{th} and 6^{th} well where infection was given one hour before the treatment. All well in the plate B was kept as non-medicated control.

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

Different doses of plant extract such as 2µl, 4µl, 6µl, 8µl and 10µl were interacted with H7N3 and H9N2 serotype of avian influenza at different time interval in 90% saturated vero cell line with constant supply of CO_2 at 37°C. It is evaluated that one hour pre-exposure, simultaneous and one hour post-exposure of TTO (10ul) to AIV-H7N3 infected vero cell line showed 0.5±1.0, 3.5±1.0 and 2.5±1.0 respectively mean standard deviation (M±SD) of HAU titer after 48 hours of incubation as showed in Fig. 2. Whereas, one hour pre-exposure, simultaneous and one hour post-exposure of TTO (10µl) to AIV-H9N2 infected vero cell line showed mean standard deviation (M±SD) of HAU titer 1.0±1.1, 5.0±2.0 and 3.0±1.15 respectively as showed in Fig. 3. Furthermore, one hour pre-exposure, simultaneous and one hour post-exposure of OLE (10µl) to AIV-H7N3 infected vero cell line showed mean standard deviation (M±SD) of HAU titer 3.0±1.1, 7.0±2.0 and 5.0±2.0 respectively as showed in Fig. 4,7. Moreover, one hour pre-exposure, simultaneous and one hour post-exposure of OLE (10µl) to AIV-H9N2 infected vero cell line showed mean standard deviation (M±SD) of HAU titer 2.5±1.0, 7.0±2.0 and 4.0±0.0 respectively as showed in Fig. 5. Similarly, untreated control group inoculated with the same dose of AIV-H7N3 and AIV-H9N2 with same conditions (M±SD) 320.0±128.0 of HAU titer. The maximum dose (10µl) of tea tree oil extract when inoculated on vero cell line displayed significantly higher anti AIV-H9 as compare to AIV-H7. Similarly, maximum dose (10µl) of Olive leaf oil extract when inoculated on vero cell line anti AIV-H7 significantly higher than AIV-H9.

DISCUSSION

Anti-viral agents have been consistently focused by the scientists in both human and veterinary sciences. The unique nature of viral replication may evade the target medicinal site and hence be successful in production of disease. Various plant extracts have been tested for antiviral activity. Over the last decade, laboratory investigation explored that numerous essential oils have potential antiviral activity. On the basis of such hypothesis two plants were selected having potential antiviral components in their leaves extracts. The extracts were evaluated for the anti influenza virus activity on vero cell line in association with their mechanism of action and least effective dosage. To determine the anti- influenza virus effect of TT leave extract, cell toxicity assay showed no CPE of TTO on Vero Cell line up to 10 µl ($p<0.05$). A. Garozzo *et al.*, 2009 reported his observation about anti-viral activity of TTO that it did not show any cytotoxic effect on MDCK cells when concentration was less than

0.025% but concentration greater than 0.050% could induce significant cellular death. This work confirms the efficiency of TTO against AIV sub-type H7N3 and H9N2 showing 10 µl of purified extract has significantly ($p<0.05$) higher anti-influenza response as compare to 8 µl, 6 µl, 4 µl and 2 µl in vero cell line as showed in Fig. 6.

According to our study prevention dose (10 µl) of TTO (Pretreatment) against avian influenza H9N2 virus on vero cell line ($p<0.05$) (1.0±1.1) just before one hour of infection is declared as optimum effective time period to counter these agents as compared to post-treatment (10 µl) (3.0±1.15) and simultaneous treatment(10 µl) (5.0±2.0) as showed in Fig. 3. G. Pourghanbari *et al.*, 2016 determine influenza virus sub-type H9N2 replication was suppressed by the diverse concentration of MOEO. Pre-infection stage show significantly effective antiviral activity of MOEO as compare to other time addition to MDCK cells. Moreover, $TCID_{50}$ in the pre-treatment group containing 0.5 and 0.005 (mg/ml) of MOEO were reduced to 0.98 and 1.9 (log 10) respectively.

Whereas, post treatment with similar dosage (10 µl) (3.0±1.15) on vero cell line showed significantly better anti-viral response to that of simultaneous treatment (5.0±2.0). The similar results were also shared by G. Pourghanbari *et al.*, 2016 who reported that in post-infection stage, the groups that received 0.5, 0.1 and 0.05 mg/ml MOEO had higher reduction in TCID50 index than other groups. Also in the simultaneous stage, the groups that receiving 0.5 and 0.1 mg/ml of the MOEO showed to be more effective against virus replication.

Extract of Olive Leafs have strong antiviral activities against hepatitis virus, bovine rhinovirus, rotavirus, feline leukaemia virus, herpes mononucleosis, and canine parvovirus (Fredrickson, 2000). In addition, OLE has also demonstrated a considerable antiviral activity against parainfluenza type 3 virus and respiratory syncytial virus. OLE exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV) and can be considered a prospective source of antiviral agents for aquaculture, which might escort to a new generation of chemicals with minor environmental impact to be carefully administered to the aquatic media (Mohammad *et al.*, 2017).

Our study showed significant ($p<0.005$) decrease in Cytopathic Effect in comparison to post-treatment (10 µl) and simultaneous treatment (10 µl). Our result resemble with Siddique *et al.*, 2012 which highlight that Pre-treatment assay showed decrease in plaque forming unit by

about (3.5 & 3) logs 10 PFU/ml in watery and alcoholic extracts respectively in comparison to the non-treated cell culture and infected only with H5N1 (4.5x106 PFU/ml).

When OLE and subtype H7N3 and H9N2 AIV was done at same time respectively represent lower HA titer as compared to pre-treatment and post-treatment while Saif *et al.*, 2005 declare that the virus titer to decreased by 2.5 and 1.5 logs as it reached $2x10^6$ & $3x10^6$ PFU/ml in case of watery & alcoholic extracts respectively in comparison to the non-treated cell culture and infected only with H5N1 ($4.5x10^6$ PFU/ml). Results also confirmed that the OLE antiviral effect was not due to its direct effect on the virion but due to the effect on the host cells by preventing or delaying cell to cell transmission as agreed with Sylvia Lee-Huang *et al.*, (2003). Pre-infection stage appears to interfere with the cell surface proteins such as masking the cell surface. Therefore, OLE and TTO can prevent the binding of the virus particle to cellular receptors. Post treatment appears to interfere with intracellular step of the virus replication. The inhibitory effect did not relate to the virus attachment to vero cells because extracts was inoculated to the cells after virus absorption phase.

The study highlight very interesting result, OLE extract is more effective on H7N3 strain as compare to H9N2 strain of AIV. Pre-Treatment Assay of TTO on H7N3 strain show significant result in contrast OLE on H9N2 strain of AIV and Pre-Treatment Assay of OLE on H9N2 strain show significant result in contrast TTO on H7N3 strain of AIV.

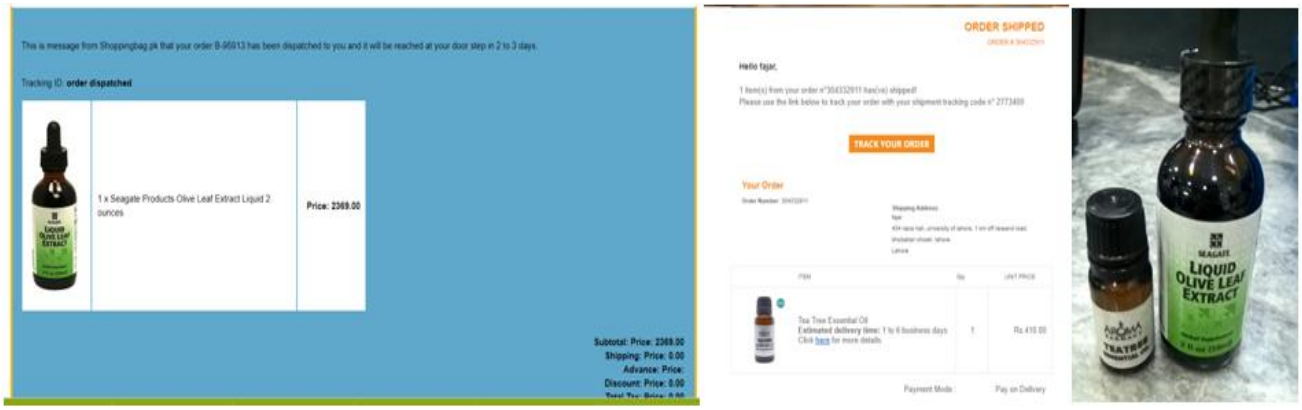

Fig. 1: Receipt of extract purchase.

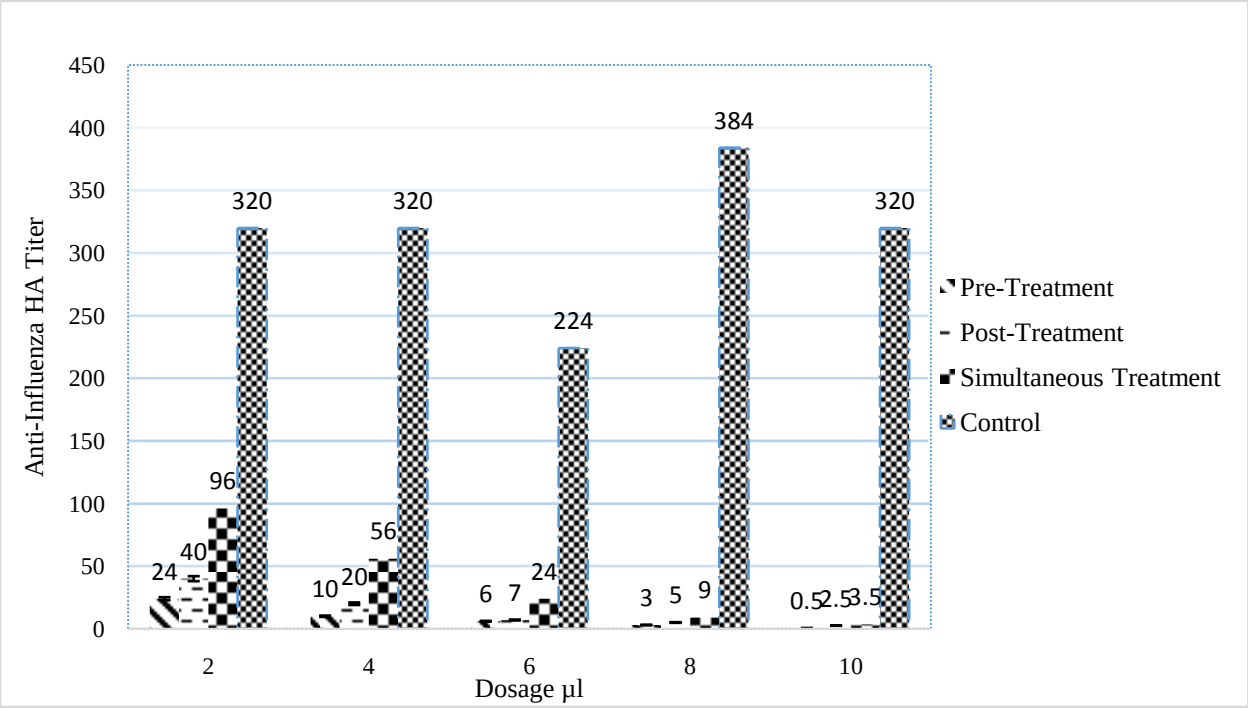

Fig. 2: Dosage associated mechanism of action of Tea Tree Oil (TTO) on AI subtype H7N3.

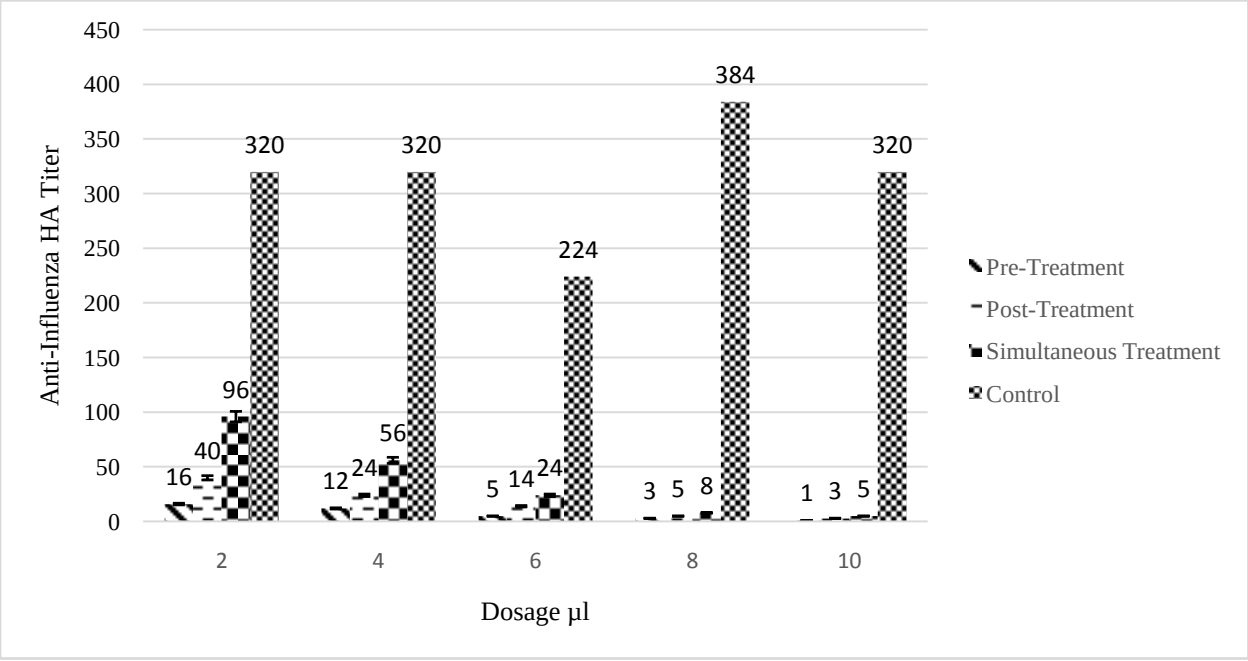

Fig. 3: Dosage associated mechanism of action of Tea Tree Oil (TTO) on AI subtype H9N2.

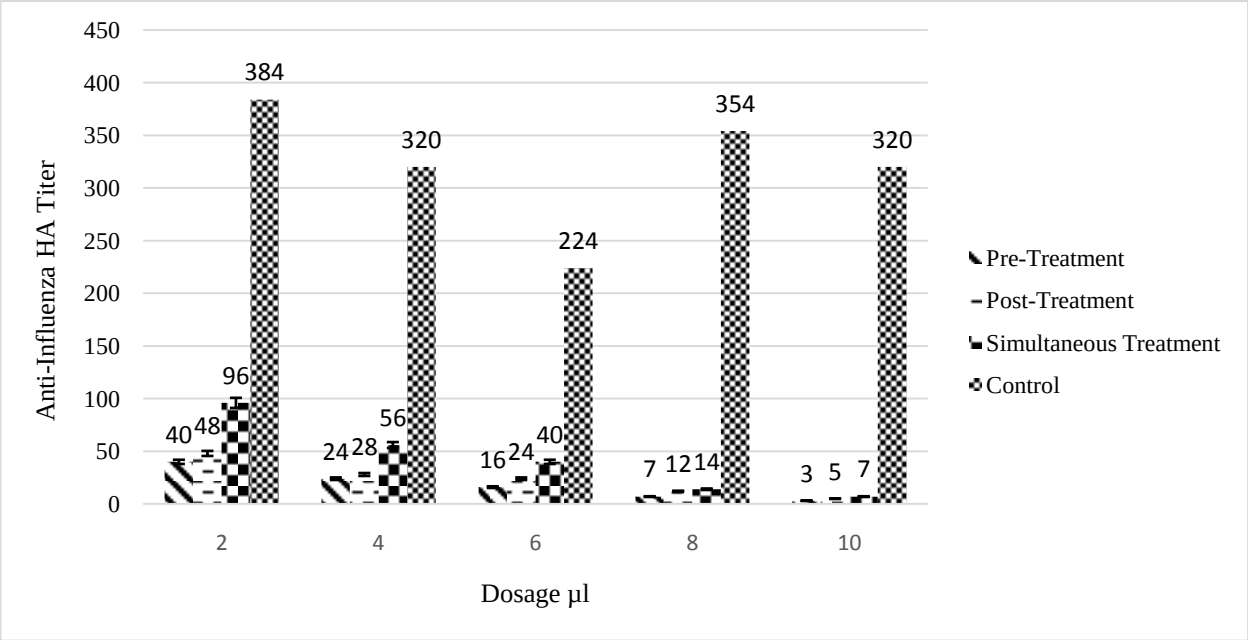

Fig. 4: Dosage associated mechanism of action of Olive Leaf Extract (OLE) on AI subtype H7N3.

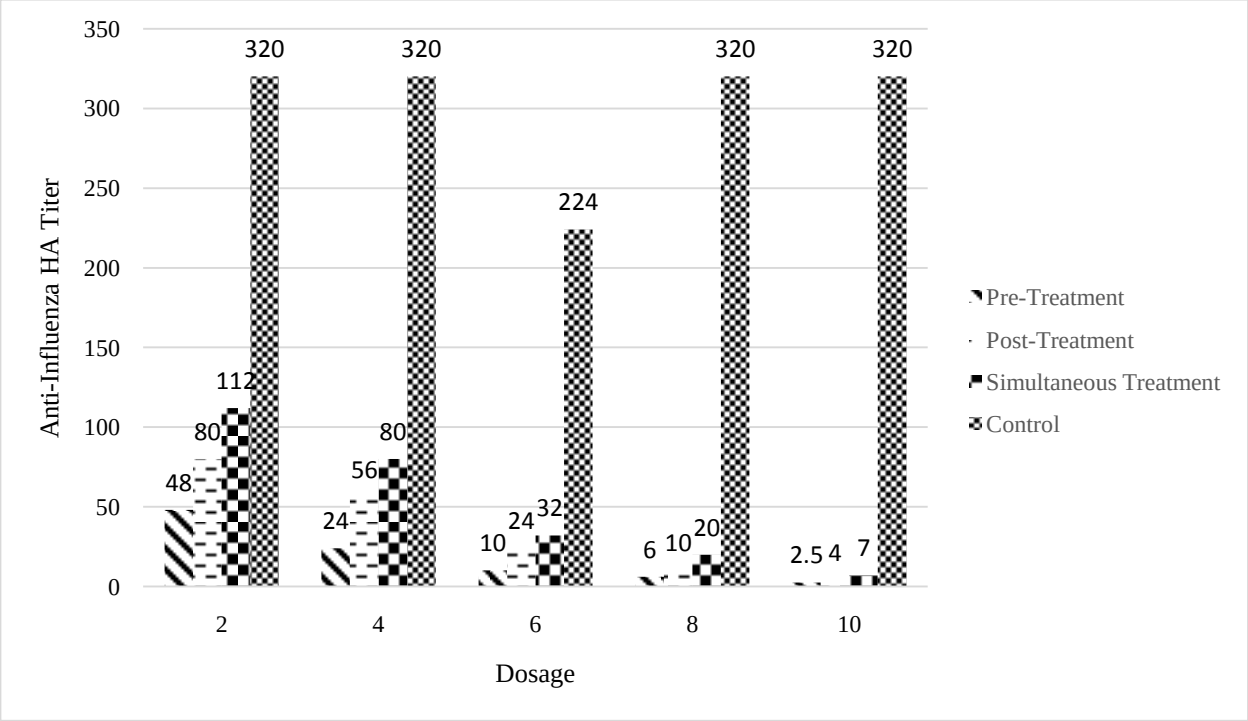

Fig. 5: Dosage associated mechanism of action of Olive Leaf Extract (OLE) on AI subtype H9N2

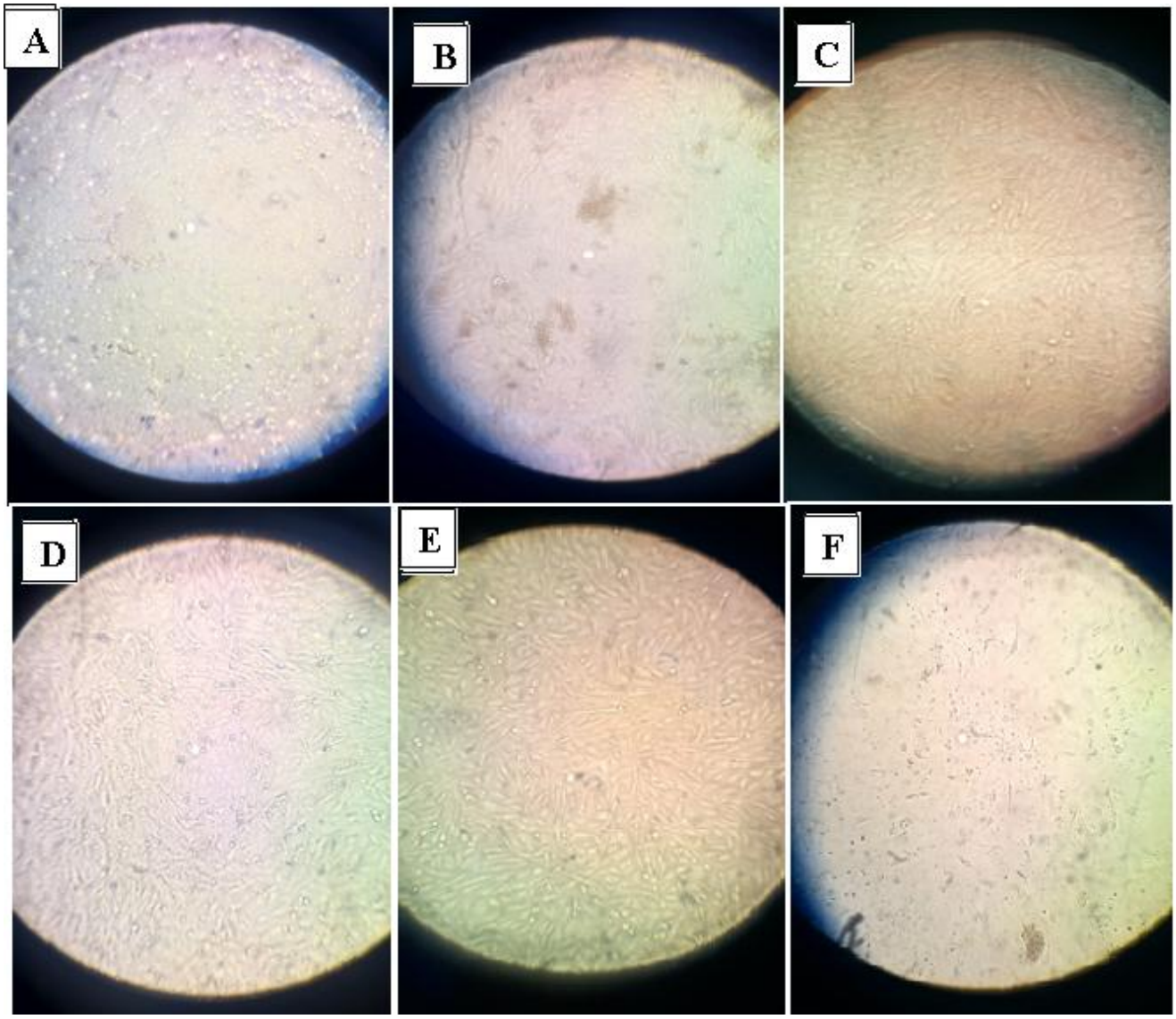

Fig. 6: Microscopic analyses of anti-influenza activity on vero cell line. A: 2 µl, B: 4 µl, C: 6 µl, D: 8 µl, E: 10 µl, F: Control

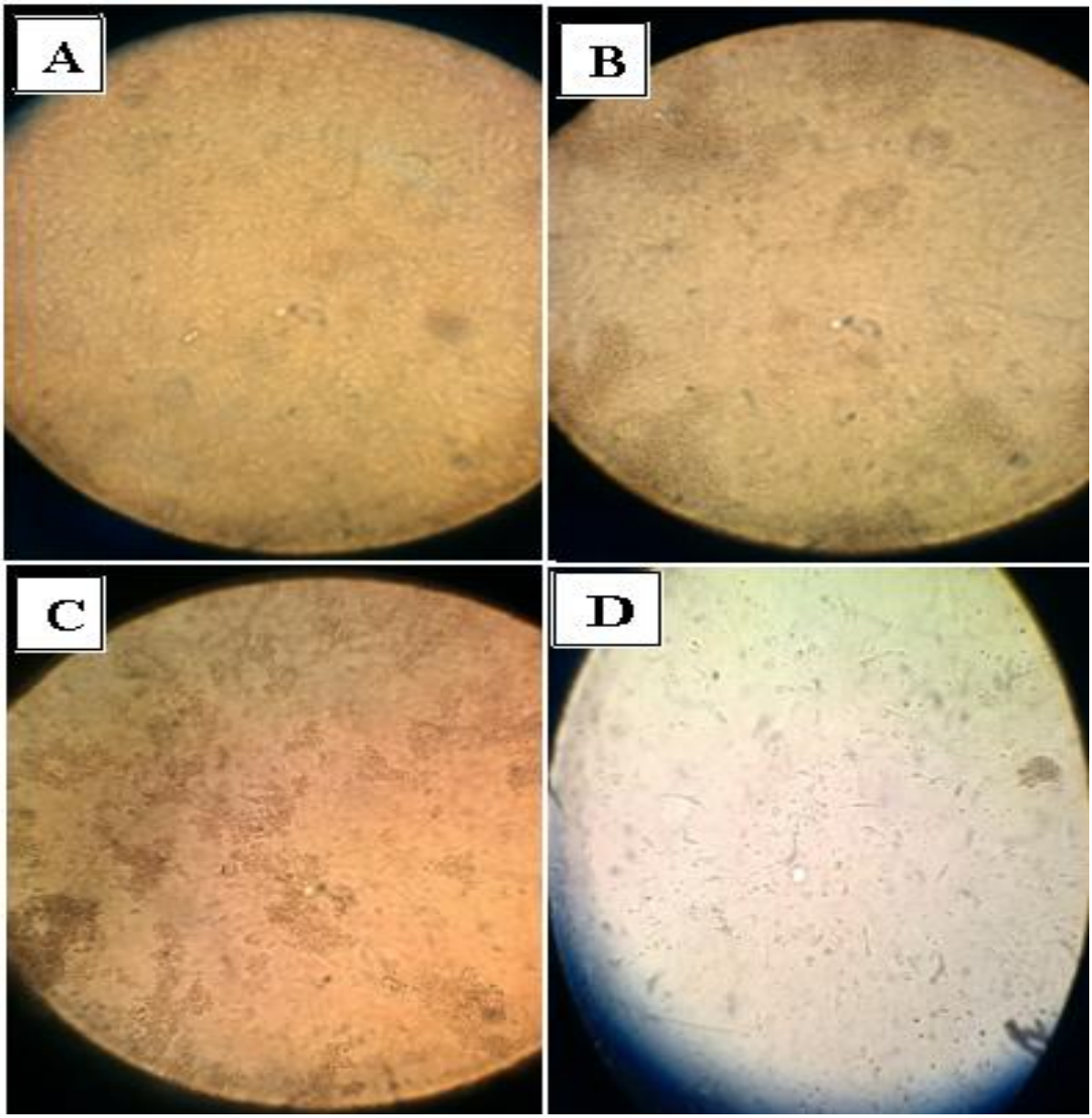

Fig. 7: Microscopic analyses of vero cell line. 1: Pre-Treatment Assay, 2: Post-Treatment Assay, 3: Simultaneous Treatment, 4: Control.

CONCLUSION

Melaleuca alternifolia (TTO) and *Olea europaea* leaves (OLE) showed significant antiavian influenza virus subtypes H7N3 and H9N2 potential at dosage of 10µl before onset of infection in vero cell line culture. It could be better to use pure extract of TTO, OLE as a preventive measure in commercial poultry to mask the mucous membrane and ultimately blocking receptor site of the viruses.

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