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In Vitro Antiviral Activity of Rooibos Tea (*Aspalathus linearis*) Leaves Aqueous Extract against Influenza Virus

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ABSTRACT

Influenza virus infection is a major public health problem and its control continues to be a challenge. The emergence of highly resistant-influenza virus strains necessitates the need to search for novel medications, especially from natural sources.
The aim of this study was to identify the anti-influenza virus activity of Rooibos tea (*Aspalathus linearis*) aqueous extract and possible mechanisms of action on different virus strains.
Anti-influenza activity was performed using standard *in vitro* assays including cell-based neutralization by inhibition of virus-induced cytopathic effect (CPE), inhibition of viral plaques and hemagglutination assay. The CPE was determined visually and by dye uptake. The time-of-addition assay was performed to determine the effect on the virus life cycle.
The results reveal that the extract showed a significant broad spectrum of anti-influenza activity against both influenza A and B with 50% inhibitory concentration (IC_{50}) of 1.3 mg/mL, while the 50% cytotoxic concentration (CC_{50}) was 40 mg/mL. The inhibitory effect of extract was identified against influenza A/PR/8/34 (H1N1), A/WSN/33 and A/HK/8/68 (H3N2) and influenza B virus strains. The virus-induced cytopathic effects were significantly reduced. Plaque assays indicated that the extract markedly reduced virus infectivity in a dose-dependent manner. Results of this study indicated that the extract directly affected virus particles and disrupts the function of virus adsorption to host cells.
Rooibos tea extract strongly inhibits influenza virus replication with a dual mode of action and may contribute to the development of a new anti-influenza virus agent.

***Keywords*:** Cytotoxicity, Influenza A virus, Plaque assay, Rooibos tea, Time of addition assay.

Introduction

Influenza or "flu" is an infectious disease caused by the influenza viruses that affects millions of people every year.[1] Influenza viruses belong to the family of orthomyxoviridae.[2] There are three types of influenza virus: A, B and C. Influenza A can infect humans and other animals, while influenza B and C infect humans only. The virus contains negative single stranded genome RNAs that are necessary for the virus to survive and replicate within host cells. Annual seasonal epidemics and occasional pandemics of influenza result in significant morbidity and mortality in both humans and animals worldwide. Furthermore, the emergence of the highly pathogenic H5N1 avian influenza virus, which was associated with a mortality rate in excess of 60% in infected individuals,[3] as well as the 2009 flu pandemic, a global outbreak of a new swine-origin strain of H1N1 influenza virus, have raised significant public-health concerns about the emergence of a potential novel highly pathogenic pandemic influenza virus strain.[4] Vaccination provides the most effective means of prophylactic antiviral therapy, while antiviral medications constitute the first line of treatment following infection.[5]

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The continuous evolution of major viral antigens, demanding the selection of vaccine strains annually. Therefore, vaccine production may not satisfy the need during an influenza pandemic. Antiviral drugs provide a valuable addition to the options available to control influenza infections. To date several synthetic drugs such as amantadine and rimantadine (M2 ion channel inhibitors) and oseltamivir and zanamivir (NA inhibitors) have proven to be clinically effective against influenza. However, due to the high mutation rate of these viruses, the emergence of drug-resistant viral strains against both classes of drugs has been reported.[6,7] Therefore, there is an urgent need for searching novel antiviral agents and approaches with numerous modes of action to control and prevent this viral disease. Studies on the anti-influenza virus activity of natural products have dramatically increased over the past several years.[8] Naturally occurring anti-viral nutrients may be of special interest because they are widely available and used as part of the diet and possibly of beneficial health effects to combat several disease models, including influenza infection.[9] It has been reported that Rooibos tea (*Aspalathus linearis*), RT, has diverse physiological and pharmacological actions such as *in vitro* anti Human immune deficiency virus (HIV) activity,[10] anti-bacterial and anti-fungal activity,[11] a potent antioxidant activity [12], anti-diabetic [13]and immune enhancement.[14] Furthermore, Rooibos has been shown to protect membrane lipids against peroxidation thereby partially preventing oxidative stress.[15] Rooibos has also been used as an anti-hypertensive, laxative, sedative, spasmolytic agent and for treatment of atherosclerosis.[16] Study showed that RT aqueous extracts of honeybush and Rooibos exhibit antimutagenic properties against aflatoxin B_1 and 2-acetylaminofluorene induced *in vitro* [17] and *in vivo* [18] mutagenesis. Recent study revealed that the Rooibos tea reduced the migration and invasion of prostate cancer cells.[19] The crude hot water RT extract is rich in flavonoids and polyphenolic substance.[20] The

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major phenolic components of the unprocessed South African herbal teas are the dihydrochalcones, aspalathin and nothofagin present in Rooibos (*A. linearis*) and the xanthone, mangiferin, eriocitrin, narirutin and flavanone, hesperidin in Honeybush (*Cyclopia intermedia*).[15, 21] However, few studies were performed about the anti-influenza activity of Rooibos tea. This study was carried out to identify *in vitro* antiviral activity of Rooibos tea leaves aqueous extract extracts on a different influenza virus strains and possible mechanisms of action.

Materials and Methods

Plant material
The crude Rooibos tea (100 g), *A. linearis* of the family Fabaceae, was collected during July 2018 from local market and authenticated by National Centre for Research, Khartoum, Sudan. A voucher specimen was deposited in the Centre with number (NCR-R.T/4/018).

Preparation of plant extract
Rooibos tea leaves (5 g) coarse powder was macerated in 100 ml of reverse osmosis water (RO) with continues shaking (Shaking Bath BW200) at room temperature for 1 day, then heated at 85°C for 10 min and cooled at room temperature. Finally it was filtered by Whatman filter paper NO 1 and stored at 4°C (to avoid deterioration) until further use.

Cell culture and virus strains
Madin–Darby canine Kidney (MDCK) cells were grow in Eagle's Minimum Essential medium (E-MEM) with L- Glutamine and phenol red and supplemented with 5% fetal bovine serum at 37°C in 5% CO_2. Influenza virus strains used were A/WSN/33 (H1N1), A/Puerto Rico/8/34 (H1N1), A/Honk Kong/8/68 (H3N2), A/Nagasaki/ HA-4/2009 (H1N1), A/ Nagasaki/ HA-58/2009 (H1N1) and B/lee/40. All virus strains were prepared from cultured supernatant of infected MDCK cells and titer was determined by Tissue Culture Infectious Dose ($TCID_{50}$/ml).[22]

The water-soluble tetrazolium salt (WST-1) assay
Madin–Darby canine Kidney cells (3×10^4/well) were seeded in a 96-well plate and incubated at 37°C for 1 day. After treated with Rooibos tea extract and/or virus infection (100 $TCID_{50}$/mL), cells were incubated for 3 days. Five mM WST-1 solution in 0.2 mM of 1-methoxy-5-methylphenaziniummethyl sulfate (Dojindo Chemicals) was added to a final concentration of 0.25 mM. The optical density (OD) was measured 3 hours later by scanning at 450 nm and 560 nm reference wavelengths in the Emax precision microplate reader (Molecular Devices). The percentage cell viability was compared with untreated controls and plotted against extract concentration, then calculate the 50% cytotoxic concentration (CC_{50}) and 50% inhibitory concentration (IC_{50}).[23] The concentration of 200 up to 1.6 nM of Zanamivir as positive control was used and it was safe to the MDCK cell with no cytopathic effect.

Crystal violet assay
Madin–Darby canine Kidney cells (3.0×10^4/well) were seeded in a 96-well plate and incubated at 37°C for 1 day.[24] After treatment with Rooibos tea extract and/or virus infection (100 $TCID_{50}$/mL), cells were incubated for 3 days. The cells were fixed with 70% ethanol for 5 min, after ethanol was removed the cells were staining with Crystal Violet (0.5%) for 5 min, The staining solution was removed and cells were washed with tap water and left to dry at room temperature. The optical density (OD) was measured by scanning at 560 nm wavelengths in the Emax precision microplate reader (Molecular Devices). The percentage cell viability was compared with untreated controls and plotted against the tea extract concentration. The 50% cytotoxic concentration (CC_{50}) and 50% inhibitory concentration (IC_{50}) were calculated. Also, the selectivity index (SI) for Rooibos tea was calculated from ration of CC_{50}/IC_{50}.

Effect of heat treatment on Rooibos tea extract
The heat stability test was carried out by taking 2 mL from stock sample at 4°C, then dispense in two 1.5 ml tubes, each tubes about 1 mL, the samples were heated at (60°C, 90°C) for 10 min using block incubator machine (B1-525), cooled to room temperature and antiviral assay was tested as described.

Plaque assay
Madin–Darby canine Kidney cells ($1.0\text{x}10^6$/ well) were seeded into 6-well plate and incubated at 37℃ for 1 day. Cells were infected with influenza A/WSN/33 virus (100 PFU/mL) with 10-fold serial dilutions of the virus in a serum-free medium for 1 h. After washing twice with PBS (-), cells were overlaid with MEM containing 0.8% agarose, 0.1% BSA, 1% 100x vitamins solution and 0.03% glutamine. Agarose gel was mixed with the Rooibos tea extracts (20, 10, 5, and 2.5) mg/mL). After 3 days of incubation, cells were fixed with ethanol: acetic acid (1:1) for 1 h at room temperature and stained with 0.5% (w/v) Amino Black 10B after removal of the overlaying agarose gel, washed with tap water and dried at room temperature.[24] The plaques were counted by visual examination. Means and standard deviations were calculated from duplicate experiments.

Time-of-addition assay
A time-of-addition experiment was performed by addition of RT at different time intervals over a 24-hours incubation period. Madin–Darby canine Kidney cells (6×10^5 cells/well) were seeded into 12-well plates and infected with virus at MOI of 0.001. RT or DMSO (0.1%, v/v) treatment was performed before, during, or after viral infection.[24] At 24 hours post-infection (p.i.), culture supernatants of infected cells with different treatments were harvested, and the virus titers were determined by PFU assay. The details procedures for each treatment were as follows: (1) Pre–treatment of cells before infection: MDCK cells were pre-treated with Rooibos tea or Dimethyl sulfoxide (DMSO) 1 mg/mL and incubated at 37°C for 2 hrs. After removal of the pre-treatment medium, the cells were wash twice with MEM (-) and infected with influenza virus. At 24 hrs p.i., cell supernatants were collected, and virus yields were determined by the PFU assay. (2) Pre-treatment of virus before virus infection: The virus (3×10^3 PFU) was pre-incubated with RT or DMSO on ice for 1 hour. The mixture of virus and RT or DMSO was then added to MDCK cells and incubated at 37°C for 1 hour. Cells were then washed twice and cultured in fresh medium for 24 hours. (3) Treatment of cells during virus infection: RT or DMSO was administered together with the virus to the cells. After infection, cells were washed twice and cultured in fresh medium for 24 hours. (4) Treatment of cells after virus infection: After virus infection, cells were treated with RT or DMSO and cultured in fresh medium for 24 hours.[24, 25]

Statistical analysis
All the obtained data were analyzed using GraphPad Prism 5.01 software. Means and standard deviations were calculated from three independent duplicated experiments. Statistical analysis was performed to compare between tested sample and control, calculated by using student t test. *P* value < 0.05 was considered significant.

Results and Discussion

Cytotoxicity of extract on MDCK Cells
Cytotoxicity effect of extract was which detect the number of dead cells in each concentration, the percentage of viable cells was compared with untreated control and plotted against compound concentrations Figure 1, there was no difference in cell shape or cell numbers could be observed compared to control. These results indicate that the extract was showed no toxic effect up to 40 mg/mL. Zanamivir was used as control in Figure 3, there was no cytopathic effect was observed up to 200 nM, this result was indicated that zanamivir is safe to the MDCK cells. The EC_{50} was 12.5 nM.

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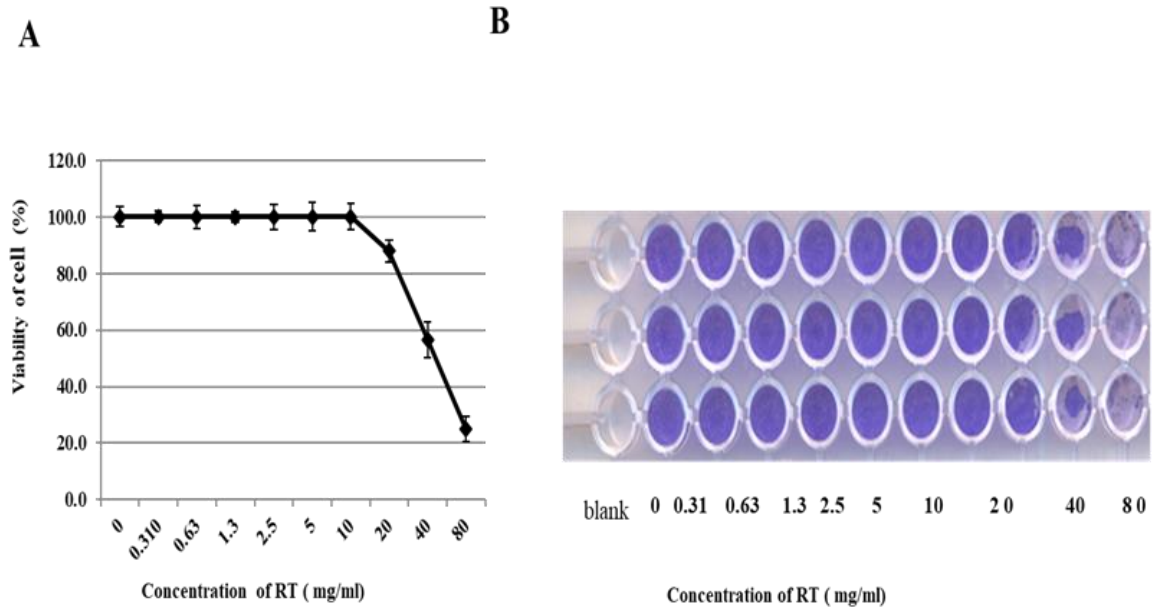

Figure 1: Effect of Rooibos tea extract on cell viability on MDCK. **(A)** Confluent MDCK cells were incubated with two-fold serial dilutions of extract. The 50% cell-inhibitory concentration (CC_{50}%) was determined. **(B)** The percentage of viable cells were compared with untreated control and plotted against compound concentrations, means and standard deviations were calculated from two independent experiments. And GraphPad Prism 5.01 software was used to calculate the 50% cytotoxic concentration (CC_{50}) that found around 40 mg/L.

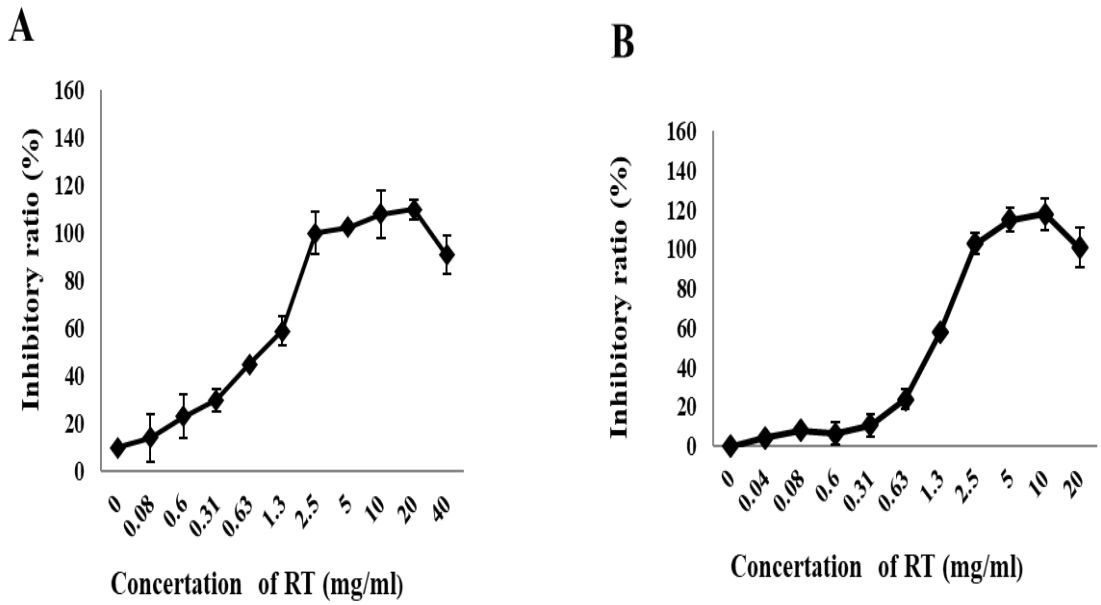

Figure 2: Effect of Rooibos tea extract on influenza A/WSN/33 virus infection by **(A)** WST-1 assay MDCK cells were treated with Rooibos tea extract and/or virus. The cells were fixed with WST-1 solution, the optical density (OD) was measured; the percentage cell viability was compared with untreated controls and plotted against the compound concentration. **(B)** Crystal violet assay MDCK cells were fixed with 70% ethanol, stained with crystal violet. The optical density (OD) was measured and the percentage cell viability was compared with untreated controls and plotted against the compound concentration. Means and standard deviations were calculated from triplicate experiments. Means and standard deviations were calculated from three independent duplicated experiments and GraphPad Prism 5.01 software was used to calculate the 50% cytotoxic concentration (CC_{50}) and 50% inhibitory concentration (IC_{50}). The selectivity index (SI) for Rooibos tea extract was calculated from ration of CC_{50}/IC_{50} = 33.

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Effect of extract on influenza A/WSN/33 virus infection by WST-1 and Crystal violet assay

The results WST-1 assay and crystal violet assay were obtained as shown in Figure 2. We found that both WST-1 and crystal violet assay were showed the same inhibitory concentration IC_{50} 1.3 mg/mL and zanamivir IC_{50} was 12.5 nM. the selective index was (CC_{50}/IC_{50} = 30.8). It was found that Rooibos tea extract exert potential anti-viral activity.

Antiviral effect of extract against influenza virus strains

The results antiviral activity of extract on the cell culture against all influenza virus strains was shown in Figure 4 and Table 1. These results suggest that Rooibos tea extract has abroad antiviral properties towards different influenza viruses. The extract was found significantly strongly active against common laboratory influenza virus strains tested such as influenza virus A/WSN/33 (H1N1), A/Puerto Rico/8/34 (H1N1), A/Honk Kong/8/68 (H3N2), and clinical isolates strains such as A/Nagasaki/ HA-4/2009 (H1N1), A/ Nagasaki/ HA-58/2009 (H1N1) and B/lee/40.

Rooibos tea extract is contains several flavonoids include flavones, flavanones, flavonols quercetin, phenolic acids and saccharides which are known to have diverse bioactivities.[26] It have been reported that acid polysaccharides and oligosaccharides from Rooibos tea leaves showed anti HIV activity and protect virus adsorption, with low cytotoxicity. It is also known to blocks HIV virion binding to the cells[10] and it also inhibits the growth of some bacterial and fungal strains.[28]

Effect of heat treatment of extract on antiviral activity

The results of extraction process on antiviral activity, as shown in Table 2 indicate that there is no any significant difference in antiviral activity with additional heat treatment of the Rooibos tea extract. Moreover, the effect of extraction time on antiviral activity of Rooibos tea was also evaluated by extracted sample in hot water and storage at 4 °C for 9 months, the anti-viral activity was done after long storage and still the extract showed antiviral activity. The results suggest that the active compound/s in Rooibos tea for antiviral might not be a protein.

Mode of action of extract against influenza A virus

Pre-incubation of cells with extract prior to infection showed no significant inhibitory effect on virus yield; however, a significant reduction in virus yield was observed when virus was pre-incubated with extract prior to infection or cells were treated with extract during or after infection (Figure 5-A), suggesting that extract may have veridical effect. Furthermore, the extract was added at different time interval on MDCK cells to determine specific time period of extract on virus replication. In comparison to DMSO treatment, extract treatment does not affect virus yield during the first 3 hours of treatment (0-3 hours); however, a significant reduction in virus yield was observed at 0-6 hours. Similarly, at 0-9 hours and 0-12 hours, the range of inhibition reached 90% and 96%, respectively (Figure 5-B). These results suggest that the extract may interfere predominantly with the late stage of the virus replication cycle, independent of its virucidal activity. Plaque assay was investigated that the extract inhibited influenza virus replication in dose dependent manner (Figure 5-C and D), 20 mg/mL showed complete inhibit of virus replication. The result of this study found similar to previous study [27], which reported that Rooibos extract was showed strong the antiviral activity of against influenza A virus.

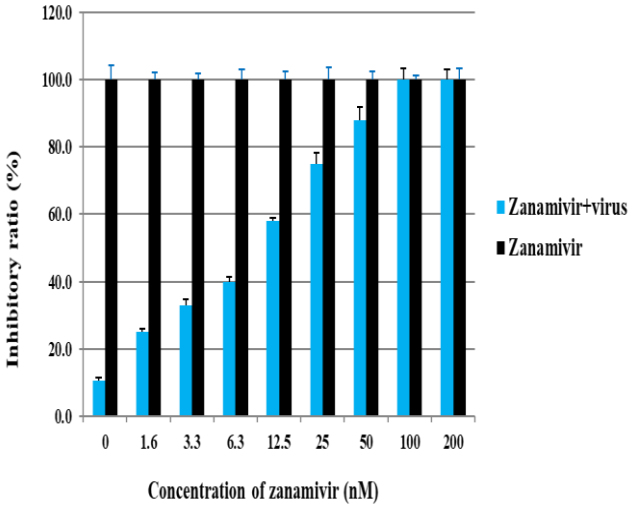

Figure 3: Cytotoxicity and antiviral effect of zanamivir. Confluent MDCK cells were incubated with absence of zanamivir or in presence of zanamivir concentration range 1.6 up to 200 nM. The 50% cell-inhibitory concentration (CC_{50}%) was not observed up to 200 nM. The percentage of viable cells were compared with untreated control and plotted against compound concentrations, means and standard deviations were calculated from two independent experiments. And GraphPad Prism 5.01 software was used to calculate the 50% of inhibitory concentration IC_{50} was 12.5 nM.

Table 1: Antiviral activity of extract against influenza virus Strains

Virus strain	IC_{50} [a] mg/mL	CC_{50} [b] mg/mL	SI [c]
A/WSN/33 (H1N1)	1.3	40	33.0
A/PR8/34 (H1N1)	2.2	40	18.2
A/HK/8/68 (H3N2)	4.4	40	9.1
HA-4pdm (H1N1)[d]	2	40	20.0
HA-58pdm (H1N1)[e]	0.6	40	66.7
B/lee/40	2.7	40	14.8

[a]IC_{50}, 50% inhibitory concentration, [b]CC_{50}, 50% cytotoxic concentration, [c] SI Selective index (CC_{50}/IC_{50}), [d] HA-4pdm, A/Nagasaki/ HA-4/2009 (H1N1), [e] HA-58pdm, A/ Nagasaki/ HA-58/2009 (H1N1)

Table 2: Effect of extraction process of extract on antiviral activity

Heating time (min)	Temperature	Activity (mg/mL)
10	60 °C	1.3
10	90 °C	1.3

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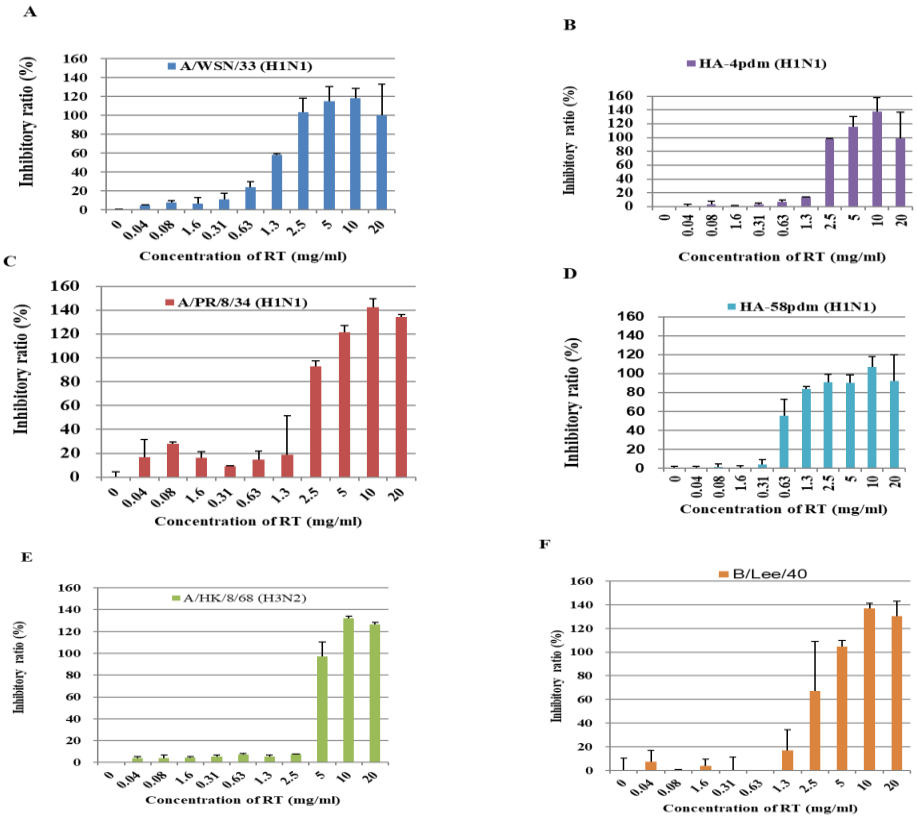

Figure 4: Anti-viral effect of Rooibos tea extract on several kinds of influenza virus strains. MDCK cells were treated with Rooibos tea extract and/or virus , left panel **A)** laboratory common strain influenza A/WSN/33 (H1N1) virus, right panel **B)** clinical isolated strain A/Nagasaki/ HA-4/2009 (H1N1), left panel **C)** laboratory common strain A/Puerto Rico/8/34 (H1N1), right panel **D)** clinical isolated strain A/ Nagasaki/ HA-58/2009 (H1N1), left panel **E)** laboratory common strain A/Honk Kong/8/68 (H3N2), right panel **F)** human influenza B/lee/40. Cells were fixed and stained with crystal Violet. The optical density (OD) was measured and the percentage cell viability was compared with untreated controls and plotted against the compound concentrations. Means and standard deviations were calculated from duplicate experiments.

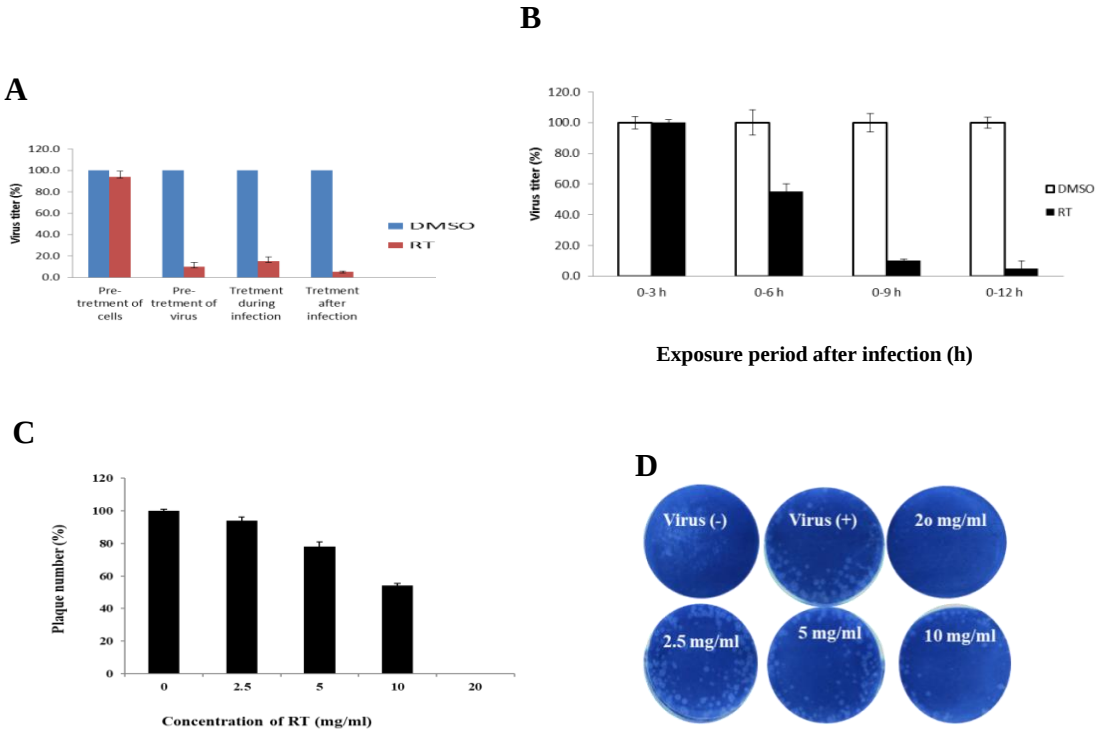

Figure 5: Mode of action of extract against influenza A virus.
MDCK cells were infected with A/WSN/33 (MOI = 0.001) and treated with Rooibos Tea (RT) and DMSO. **(A)** Extract addition before, during, after virus infection. **(B)** Infected cells were exposed to RT for different periods of time after infection (0-3 hrs, 0-6 hrs, 0-9 hrs and 0-12 hrs p.i.). **(C)** The virus titers from the extract-treated cells present as a percentage of the control (0.1% (v/v) DMSO). Values represent the mean of three independent experiments, and error bars show the standard deviation of the mean. **(D)** MDCK cells were infected with influenza A/WSN/33 virus with serial 10-fold dilutions. Agarose gel was mixed with the RT extract (20 10, 5, and 2.5) mg/mL, then cells were fixed with ethanol: acetic acid and stained with Amino Black.

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Conclusion

This study showed that Rooibos tea aqueous extract have strong antiviral activity with low inhibitory concentration (IC_{50}, 1.3 mg/mL) and less toxicity (CC_{50}, 40 mg/mL). Time of addition and plaque assay proved that the extract interfere predominantly with the virus particle and induced virucidal activity. This anti-influenza activity of the tested plant could optimize its efficacy against respiratory tract viral infection stains. Further studies should perform on an *in vivo* for efficacy and toxicity of the plant extract and should elucidate the active principles responsible for anti-influenza activity.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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