

# COMPARATIVE ANTI-INFLUENZA POTENTIAL OF *MORINGA OLEIFERA* LEAVES AND AMANTADINE INVITRO

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## ABSTRACT

**Introduction:** Influenza is an extremely communicable respiratory disease with widespread morbidity and mortality. Amantadine is a standard drug used against influenza virus and blocks its replication by preventing uncoating of the virus. *Moringa oleifera* is an herbal plant with extensive therapeutic effects and has established antiviral potential against Equine herpes virus 1 and Herpes simplex virus 1.

**Objective:** Comparison of cytotoxic and anti-influenza potential of *Moringa oleifera* leaves and amantadine.

**Methods:** This project was done in the department of Pharmacology Shaikh Zayed Postgraduate Medical Institute in collaboration with University of Veterinary and Animal Sciences (UVAS), Lahore. Influenza virus (H9) was obtained from Quality Operational Lab (UVAS) followed by its identification by using standardized method of haemagglutination inhibition assay and quantification by haemagglutination assay and tissue culture infective dose (TCID<sub>50</sub>). Ethanolic extract of *Moringa oleifera* leaves (EEOMOL) was produced using soxhlet apparatus. Fibroblast cell line established from chicken embryo was used for testing of cytotoxic and anti-influenza potential of EEOMOL and amantadine using MTT assay.

**Results:** EEOMOL has higher CC50 (100ug/ml) value compared to that of amantadine (50ug/ml). Regarding antiviral activity, EEOMOL showed significantly better ( $P < 0.05$ ) anti influenza activity (0.78µg/ml to 100ug/ml) as compared to that of amantadine (12.5ug/ml to 50ug/ml).

**Conclusion:** These findings conclude that *Moringa oleifera* leaves possess wider range of anti- influenza activity compared to amantadine.

**Key words:** Influenza virus subtype H9, Amantadine, ethanolic extract of *Moringa oleifera* leaves, MTT assay.

## INTRODUCTION

Influenza is a respiratory viral disease that spreads around the world as seasonal epidemics and is caused by segmented RNA enveloped influenza viruses from the Orthomyxoviridae family. Infectivity of these viruses is facilitated by viral glycoproteins<sup>(1)</sup>.

The influenza A virus (H9) is now considered wide spread in poultry and has ability to infect human beings<sup>(2)</sup>. This subtype has shown a vast host range and can infect mammals including swine and humans. Humans infected by H9N2 are reported from China and Hong Kong<sup>(3,4)</sup> but transmission of this virus from one species to other is still poorly understood<sup>(5)</sup>.

Amantadine is a standard anti-influenza drug which is derived from adamantane. It is effective only against influenza A virus with its ability to block M

proton channel of the virus which is important for its uncoating<sup>(6)</sup>. Use of this drug in patients is associated with many CNS side effects and can rarely cause Stevens Johnson syndrome and livedoreticularis<sup>(7)</sup>. Influenza A viruses (H1, H3, H9) have shown increased level of resistance against amantadine because of the mutations in M2 gene<sup>(8)</sup>.

Natural plants are extensively used as herbal medicines because they are rich in bioactive principles<sup>(9)</sup>. *Moringa oleifera* (Sohanjna) from Moringaceae family is a small, rapidly growing, evergreen tree cultivated in Pakistan and West Asia. All parts of the plant have therapeutic potentials, but leaves are the greatest nourishing part, as these are rich in vitamins, minerals, antioxidants, nitrile glycosides, proteins and phenolics.<sup>(10,11)</sup>

*Moringa oleifera* is investigated to possess anti-diabetic<sup>(12)</sup>, hypolipidemic<sup>(13)</sup>, hepatoprotective<sup>(14)</sup>, analgesic, anti-inflammatory<sup>(15)</sup> and widely recognized antibacterial<sup>(16)</sup> properties. This plant has also shown antiviral activity against equine herpes virus I<sup>(17)</sup>, herpes simplex virus I<sup>(18)</sup> and Foot and mouth disease virus<sup>(19)</sup>. Researched antiviral activity of this extract is because of its alkaloids, saponins, glycosides, niazimin, phenolic compounds and terpenoids<sup>(20)</sup>. Due to the projected potential antiviral activity of *Moringa oleifera*, it is proposed that it can also possess anti-influenza activity.

There are increased chances of influenza virus (H9) pandemics, it is the need of the hour to establish a new drug which can combat the failure of existing anti-influenza drugs. This project was proposed to compare cytotoxic and anti-influenza potential of ethanolic extract of *Moringa oleifera* leaves and amantadine. Anticipated outcome could be the establishment of a prospective anti-influenza drug against H9 virus having low price and easy availability.

## MATERIALS AND METHODS:

This research project was accepted by ethical review board of Shaikh Zaid Postgraduate Medical institute (SZPGMI). It was conducted in SZPGMI in collaboration with Quality Operational Lab, University of Veterinary and Animal Sciences, Lahore.

### Preparation of ethanolic extract:

Fresh *Moringa oleifera* leaves were acquired from Lahore (Pakistan) followed by their identification from herbarium of The University of Punjab, Lahore, Pakistan. These leaves were washed, air dried and ground to produce powder. 100 grams of the powdered leaves were extracted with 500ml of 95% ethanol using soxhlet apparatus (CG-1368). This extract was filtered with sterilized Whatman's filter paper followed by its drying to a semisolid paste in rotary evaporator (Stuart RE-300).

### Preparation of stock solution and dilutions:

Stock solutions of EEOMOL and amantadine were made by dissolving 8 mg of the chemical in 10 ml of cell culture (M-199) to obtain strength of 800µg per ml. Serial two fold dilutions of both stock solutions were carried out to get solutions having concentrations of 400 µg/ml till 0.02 µg/ml. Before use, all these dilutions were filtered through Minisart (Sartrious) 0.22 µm syringe filters.

### Virus stock:

Influenza virus of H9 subtype was procured from Quality Operation Laboratory (ISO certified), University of Veterinary and Animal Sciences, Lahore followed by its identification by haemagglutination inhibition assay<sup>(21)</sup>. The quantification of virus was performed by haemagglutination assay and tissue culture infective dose (TCID<sub>50</sub>).

### Cell line:

Fibroblast cell line was developed from a six to nine days old chicken embryo obtained from a local hatchery. After separation of head, legs, wings and visceral organs, the remaining tissue was triturated and poured in a flask containing 0.2% trypsin followed by neutralization of this trypsin with fetal bovine serum (FBS). Centrifugation of filtered cell suspension was done in a sterilized glass test tube at 1500 rpm for 3 minutes. After removal of supernatant the pellet containing cells was redissolved in 3ml of growth medium. The live to dead ratio of cells was counted by using haemocytometric (dye exclusion) method. The cell suspension with growth medium was diluted up to 2x10<sup>5</sup> live cells per ml. Incubation of this suspension was done in a flask at 37°C for 48 hours with 5% CO<sub>2</sub> in the incubator. Percentage viability of cells was calculated by using the following formula

$$\% \text{ Viable cells} = \frac{\text{Number of viable cells / ml}}{\text{Total number of cells / ml}} \times 100$$

### Propagation of cell line:

Cells of confluent monolayer in the flask were cleaned with PBS and detached by 5ml of 0.25% trypsin-EDTA. Disaggregation of cells was done by gentle shaking and tapping of the flask after incubation for 5 minutes at 37°C. Washing of clumped cells with 5ml cell culture media and gentle passing through a pipette was done to break cells. Centrifugation of these suspended cells was

done at 200 x g in 15ml conical tube for 5 minutes. Required dilution of 10<sup>5</sup> cells per ml was obtained by redispersion of cells in the growth medium after removal of the supernatant. Every well was filled with 100µl cell suspension followed by incubation at 37°C for 72 hours in 5% CO<sub>2</sub>. Cell monolayer formation was confirmed by monitoring of well plates under inverted microscope (Olympus CK40, Japan).

**Cytotoxic assay:**

Cytotoxic potential of dilutions was obtained by using confluent monolayer of 80 to 90% viable cells. Growth medium of the 96 well plates was removed followed by washing of cells with sterilized PBS. 100µl of freshly prepared culture medium was added to each well. 200ul of the stock solution was poured in the first well followed by two fold dilutions up till 0.02µg/ml. incubation of these plates was done for 48 to 72 hours at 37° C with 5% CO<sub>2</sub>. In positive control fibroblast cells with culture media were taken while in negative control 20% DMSO was taken along with fibroblast cells and culture media.

**Antiviral assay:**

In antiviral assay 96 well plates were prepared in the same way as were in the cytotoxic assay. In this assay, influenza virus was added to all the dilutions at dose of 10<sup>-7.5</sup> TCID 50 and incubated for 15 minutes at 4° C. After incubation, mixture was poured on the cells and plates were incubated for 48 to 72 hours at 37°C with

5% CO<sub>2</sub>. Fibroblast cells with culture media were taken as positive control while fibroblast cells with culture media and influenza virus were taken as negative control.

**MTT assay:**

MTT assay was used to quantify viable cells. For this purpose, drug treated culture medium was removed from all wells of incubated plates and fresh culture medium was added after rinsing the cells with phosphate buffer saline. This was followed by addition of 100 µl of 0.5% MTT solution to all the wells. Incubation of plates was done for 4 hours at 37° C followed by removal of MTT dye and cell culture media. After this 100 µl of DMSO (10%) was poured in each well and incubation of plates was done at 37°C for 2 hours. Enzyme-linked immunosorbent assay was used to calculate OD values at a wave length of 570 nm. Cell survival Percentages at all concentrations were calculated by using the following formula

$$\text{Cell Survival percentage} = \frac{\text{Mean Optical Density of test} - \text{Mean Optical Density of negative control}}{\text{Mean Optical Density of positive control}} \times 100$$

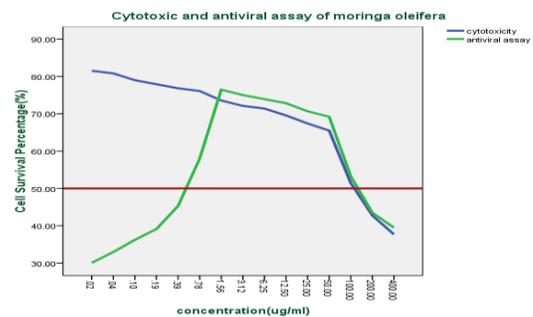
**Statistical analysis**

SPSS IBM statistics 20 was used to analyze the data. Results of drugs were presented as mean ± standard deviation and were evaluated as cell survival percentages. These percentages were compared using ANOVA. Post hoc Tukey's test was applied where required. Concentrations having cytotoxic and anti-influenza potential were compared by chi square test. P value ≤ 0.05 was considered statistically significant.

**RESULTS****Cytotoxic and antiviral assay of ethanolic extract of *Moringa oleifera*:**

In cytotoxic assay, cytotoxic concentration 50 (CC50) was 100 µg/ml as cell survival percentage decreased to 50% at this concentration. Twelve concentrations (0.02 µg/ml to 50 µg/ml) were below CC50 and safe while two concentrations (200µg/ml and 400µg/ml) were above CC50 and cytotoxic to fibroblast cells.

In antiviral assay, effective concentration 50 (EC50) was 0.78µg/ml as cell survival percentage was almost 50% at this concentration. Non cytotoxic concentrations below EC50 (0.02 µg/ml to 0.39 µg/ml) lacked antiviral potential while those above EC50 (1.56 µg/ml to 100 µg/ml) possessed antiviral activity.



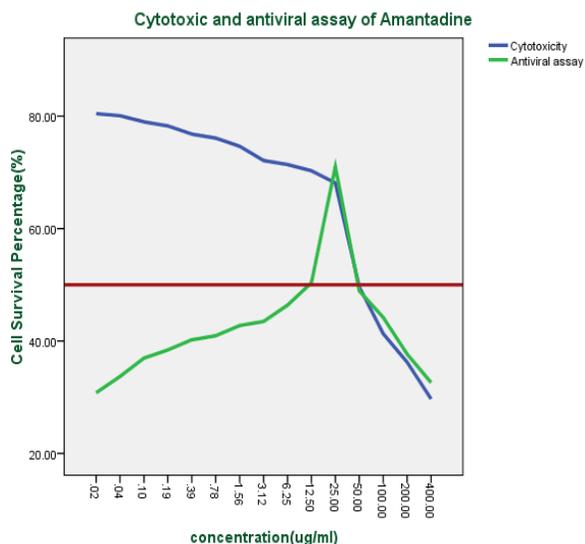
**Fig 1:** Relationship of increasing concentration of EEOMOL to cell survival percentage. In cytotoxic assay cell survival percentage is above 50% between 0.02µg/ml upto 100µg/ml, so these concentrations are proposed safe. In antiviral assay non cytotoxic concentrations between 1.56 µg/ml upto 100 µg/ml have shown more than 50% cell survival percentage so they are considered to have antiviral activity.

**Cytotoxic and antiviral assay of Amantadine:**

In cytotoxic assay, cytotoxic concentration 50 (CC50) was 50µg/ml. Eleven concentrations (0.02µg/ml to 25µg/ml) were below CC50 and safe for cell line while three concentrations (100 µg/ml to 400 µg/ml) were above CC50 and cytotoxic to fibroblast cell line.

In antiviral assay, effective concentration 50 (EC50) was 12.5 µg/ml. Non cytotoxic concentrations

below EC<sub>50</sub> (0.02 µg/ml to 6.25 µg/ml) lacked antiviral potential while those above EC<sub>50</sub> (25 µg/ml to 50 µg/ml) possessed antiviral activity.



**Fig 2:** Relationship of increasing concentration of amantadine to cell survival percentage.

In cytotoxic assay cell survival percentage is above 50% at concentrations of drug between 0.02µg/ml upto 25µg/ml, so these concentrations are proposed safe. In antiviral assay non cytotoxic concentrations between 0.78 µg/ml upto 100 µg/ml have shown more than 50% cell survival percentage so they are considered to have antiviral activity.

## DISCUSSION

Recent influenza pandemics and increasing drug resistance are the reasons for the development of newer anti-influenza drugs. Amantadine is effective against influenza virus due to its attachment to M protein channel of the virus (6). In the recent influenza epidemics extensive amantadine resistant strains have emerged(8).

Many herbal plants possess therapeutic effectiveness due to the presence of wide range of phytochemicals. Among them, *Moringa oleifera* is an herbal plant with extensive therapeutic activities (10). This plant possesses antiviral activity against different DNA and RNA viruses (17-19).

This study was done to compare cytotoxic and anti-influenza activity of ethanolic extract of *Moringa oleifera* leaves (EEOMOL) with that of amantadine. For cytotoxic potential respective CC<sub>50</sub> values were compared while for antiviral activity EC<sub>50</sub> values were used. EEOMOL has higher CC<sub>50</sub> (100ug/ml) value compared to that of amantadine (50ug/ml) making

amantadine more cytotoxic among them. These results of the extract are similar to a study in which concentrations above 100 µg/ml were established cytotoxic to COR L-23, PC3 and 10FS cells (22). Results of CC<sub>50</sub> of amantadine are similar to a project in which its CC<sub>50</sub> on MDCK cells came out to be 0.32ug/ml (23).

In present study, EEOMOL has shown a wider range of in-vitro anti-influenza potential as compared to that of amantadine. This wider range is because of low EC<sub>50</sub> (0.78µg/ml) and high CC<sub>50</sub> (100ug/ml) of the extract. Similar results regarding antiviral potential were seen in another project where antiviral potential of *Moringa oleifera* was seen at 100ug/ml against HSV(18). Amantadine showed lower range of anti-influenza potential (12.5ug/ml to 50ug/ml) due to larger EC<sub>50</sub> (12.5ug/ml) and smaller CC<sub>50</sub> (50ug/ml). These results of amantadine are in accordance to a former project where its EC<sub>50</sub> against H1N1 strain of influenza was 0.21ug/ml (23).

Due to narrow antiviral range and increasing resistance against amantadine there is dire need to establish a new anti-influenza drug with wider antiviral range and less resistance. As *Moringa oleifera* has shown anti-influenza potential, its use will be a breakthrough to fight future outbreaks of influenza virus H9.

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