ISENOLIC®, THE NOVEL OLIVE (*Olea Europaea* L) LEAVES EXTRACT IMPAIRS INFECTIVITY OF INFLUENZA A IN MDK-SIAT1 CELLS.

Salamanca A[1], Hortelano P[1], Prodanov M[2], González-Hedström D[1], Inarejos-García AM[1].

1. Pharmactive Biotech Products SL, Parque Científico de Madrid, Universidad Autónoma de Madrid, Spain
2. Instituto de Investigación en Ciencias de la Alimentación CIAL; CSIC-Universidad Autónoma de Madrid, Spain.

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Introduction

Flu has a long history of causing morbidity and mortality in the human population through routine seasonal spread and global pandemics. The high mutation rate of the RNA genome of the influenza virus, combined with assortment of its multiple genomic segments, promote antigenic diversity and new subtypes, allowing the virus to evade vaccines and become resistant to antiviral drugs.

Furthermore, the antiviral drugs can cause a wide range of adverse events to the patients, and that is why continuing research for new anti-influenza therapy using novel targets and creative strategies is needed. To this aim, olive (Olea europaea) leaves extracts have been recently studied as novel antimicrobial without substantial side effects.

Most of the olive leaves studies describe the effect of oleuropein as main antimicrobial molecule, but this molecule is poorly absorbed due to its large size and planar configuration. Elenolic acid is also found in olive leaves, it shows higher bioavailability and specific antimicrobial activity as antiviral, proven in humans, birds and mammals targeting various virus strains associated with respiratory conditions such as influenza A and B and parainfluenza 1, 2 and 3.

Isenolic®, the first commercial olive leaves extract enriched in elenolic acid analysed by high performance liquid chromatography, may have potential antiviral effect against influenza viruses.

Objectives

Evaluation of the potential antiviral effect of Isenolic® in influenza viruses.

Materials and Methods

- *In vitro* cytotoxicity determination

Cytotoxicity analysis of Isenolic® was performed in MDK-SIAT1 cells, using vehicle (ddH2O+0.01% CHCl3) and DMSO as experimental controls. Isenolic® preparations at concentrations from 6 to 1200 µg/mL were tested in 24 hours periods of exposure time. Cell viability was evaluated by the MTT tetrazolium reduction assay.

- *In vitro* antiviral activity determination

Antiviral activity evaluation of Isenolic® was performed in MDK-SIAT1 cells infected with Influenza A (H3N2) virus, using Oseltamivir as experimental control. MDK-SIAT1 cells were exposed to Isenolic® at concentrations from 6 to 100 µg/mL for 1 hour. Subsequently, cells were exposed to the virus at MOI: 1 for 1 hour (previously determined non-cytotoxic concentration). After 48 hours of infection, neuraminidase activity was measured by fluorometric assay as an indicator of influenza infectivity.

- *In vitro* viral cytopathic effect preventive action determination

Viral cytopathic effect preventive action of Isenolic® was tested in MDK-SIAT1 cells infected with Influenza A (H3N2) virus, using Oseltamivir as experimental control. MDK-SIAT1 cells were exposed to Isenolic® at 100 µg/mL for 1 hour. Subsequently, cells were exposed to the virus at MOI: 2 for 1 hour (previously determined cytopathic concentration). After 48 hours of infection, cell viability was evaluated by the MTT tetrazolium reduction assay.

Results

- *In vitro* cytotoxicity determination (figure 2)

Cytotoxic concentration (CC_{50}) for Isenolic® was estimated to be between 120 and 300 µg/mL. Cell viability was superior to 90% for concentrations from 6 to 60 µg/mL, starting to decrease at 120 µg/mL where it was reduced to 70%.

- *In vitro* antiviral activity determination (figure 3)

Inhibition concentration (IC_{50}) for Isenolic® was established at 60 µg/mL. At 100 µg/mL, inhibition rate increases to 65%. For concentrations of 6 and 60 µg/mL, inhibition rates were lower than 10%. Oseltamivir showed a maximum inhibition rate of 90%.

- *In vitro* viral cytopathic effect preventive action determination (figure 4)

Influenza infection at MOI:2 reduces cell viability to less than 30%. Isenolic® pre-treatment preserves cell viability, resulting in 73% of cell viability. Oseltamivir showed a 94% of cell viability.

Discussion

Isenolic® exerts a significant inhibitory effect of viral activity in MDK-SIAT1 cells infected with Influenza A (H3N2) at a concentration well below its cytotoxic concentration. The maximum antiviral activity rate of Isenolic® observed in this assay was 65%, at a dose of 100 µg/mL. Although lower, antiviral activity of Isenolic® is quite comparable to that observed for the gold standard treatment, Oseltamivir. Isenolic® was also efficient in protecting cells from viral cytopathic effect. Dose of 100 µg/mL preserves cell viability at 73% rate, being again near to that of Oseltamivir.

Several plant extracts with antiviral activity have proven their efficacy in influenza infections treatment. Further investigation and *in vivo* assays need to be done to confirm the potential of Isenolic® for phytotherapic treatment of Influenza A virus infection.

Conclusions

- Pretreatment with Isenolic® inhibits Influenza A activity and preserves cells form its cytopathic effect.
- Isenolic® acts as an effective antiviral agent impairing influenza A infectivity in MDK-SIAT1 cells.

This study shows promising results for the antiviral effect of Isenolic® as potential treatment to conventional flu treatment.

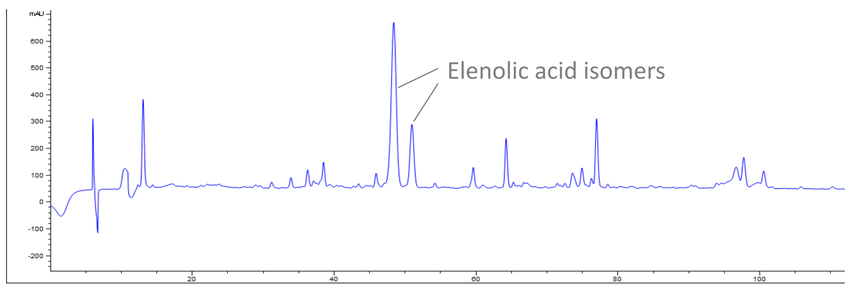

Figure 1. Characterization of Isenolic®. High performance liquid chromatography (HPLC-DAD) was performed to identify elenolic acid isomers in Isenolic®. HPLC chromatogram was registred at 242nm.

In vivo study

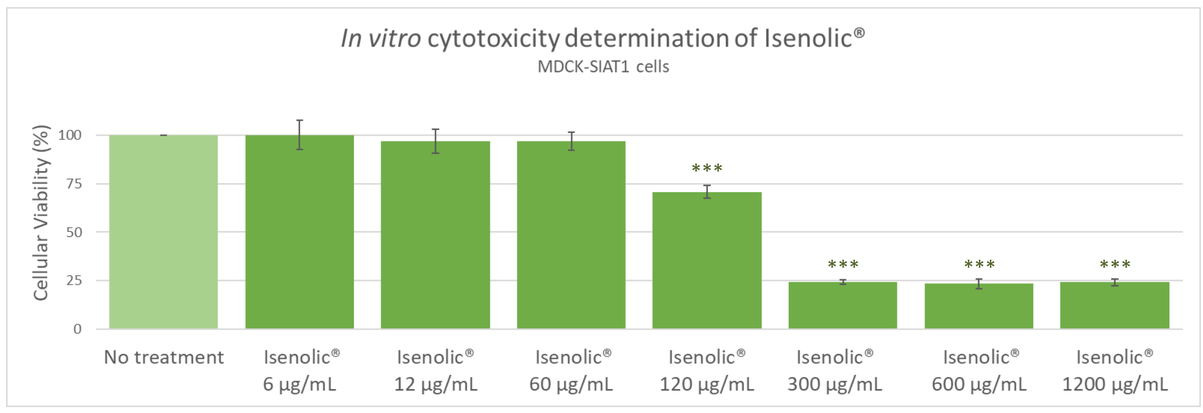

Figure 2. *In vitro* cytotoxicity determination of Isenolic®. Variation of MDCK-SIAT1 cells viability following incubation with Isenolic®, measured by MTT tetrazolium reduction assay. Data shown represent average values and standard deviation of 3 independent experiments. Statistical analysis was performed applying Student t-test to compare to no treatment group (***p<0,001).

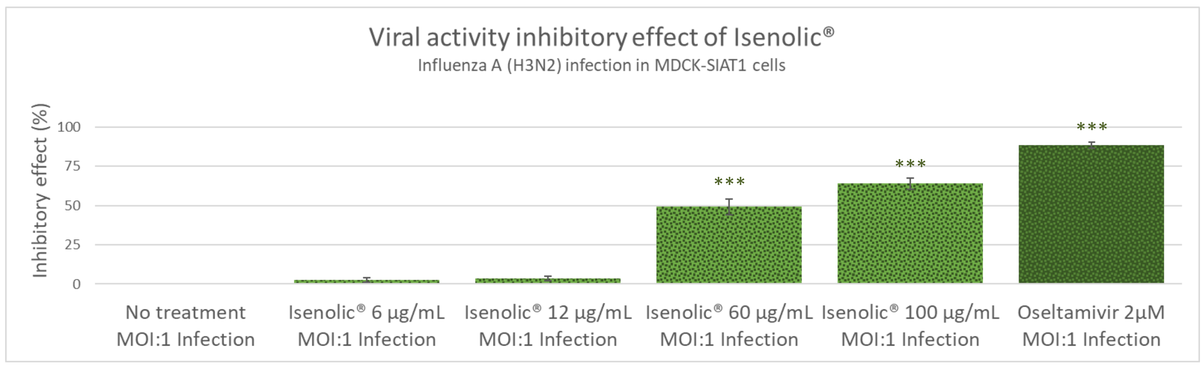

Figure 3. *In vitro* antiviral activity determination of Isenolic®. Inhibitory effect of Isenolic® pre-treatment against Influenza A (H3N2) activity in MDCK-SIAT1 cells. (MOI:1, previously determined non-cytotoxic concentration). Viral activity was measured by neuraminidase activity fluorometric assay. Gold-standard treatment, Oseltamivir, was used as reference. Data shown represent average values and standard deviation of 3 independent experiments. Statistical analysis was performed applying Student t-test to compare to no treatment group (***p<0,001).

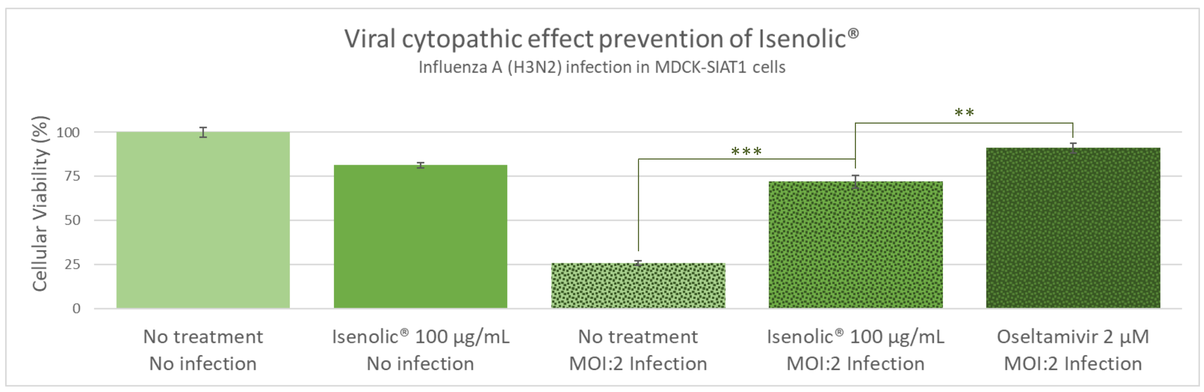

Figure 4. *In vitro* viral cytopathic effect preventive action determination of Isenolic®. Variation of MDCK-SIAT1 cells viability following Isenolic® pre-treatment and subsequent Influenza A (H3N2) infection (MOI:2, previously determined cytotoxic concentration), measured by MTT tetrazolium reduction assay. Gold-standard treatment, Oseltamivir, was used as reference. Data shown represent average values and standard deviation of 3 independent experiments Statistical analysis was performed applying Student t-test to compare to no treatment group (**p<0,01; ***p<0,001).

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