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TECHNICAL REPORT on "MAG OXYGEN (PLT_GEN_IONI) device - PlatiuMed Srl: EVALUATION OF THE ANTIMICROBIAL ACTIVITY".

Experimental setting

The antimicrobial activity of MAG OXYGEN (PLT_GEN_IONI) device was tested against bacteria and viruses; detail on the experiments and results are herein reported. For biosafety reasons (containment of pathogenic microorganisms), experiments were performed using the instrument inside a plastic box (LxWxH being 40x40x20.5 cm).

The instrument was always turned on according to the producer instructions and, after 15 minutes microorganisms were introduced inside the box (containing a modified atmosphere due to the device function) to evaluate the microbicide activity. Control samples were exposed to standard atmosphere.

Section 1: Antibacterial activity

Bacterial strains employed for this study included:

- *Escherichia coli* ATCC 25922, a Gram-negative bacterium;
- *Staphylococcus aureus* ATCC 6538, a Gram-positive bacterium.

The selected bacterial strain was grown and maintained in Trypticase soy broth or agar at 37 °C. At the time of the experiments, $1x10^7$ colony-forming units (CFU)/mL were prepared. A volume of 100 µL ($1x10^6$ CFU/mL) was deposited on sterile filter papers (1 cm^2).

The MAG OXYGEN (PLT_GEN_IONI) device was turned on 15 minutes prior to the introduction of the microorganisms into the plastic box. Thus, filter papers containing the bacterial cultures were introduced into the box and exposed for 15 or 30 minutes. Control samples were prepared as previously described and incubated for 15 or 30 minutes under laminar flow and sterile conditions. At the end of the incubations, bacteria were recovered from the filter papers using sterile culture media, appropriately diluted, and seeded on microbiological culture plates to assess microbial growth. The plates were incubated at 37 °C and colonies were observed every 24 hours for 72 hours. Microbial colonies, indicative of microbial growth, were counted. Results are reported as killing percentage calculated over control samples. Data are reported as mean±st err of 3 independent experiments.

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As reported in Table 1 and Figure 1, incubation for 15 minutes halved the bacterial load in the culture of *E. coli*. Indeed, the treatment killed 47.18±2.42% of the Gram-negative bacteria compared with the control non-treated (nt) samples (0% killing). In cultures of *S. aureus*, the killing percentage after 15 min treatment was 26.53±2.81%. The experiments were the incubation time was 30 minutes resulted in a greater extent bacterial killing of the MAG OXYGEN (PLT_GEN_IONI) device. In fact, it was observed that 94.87±0.15% of the cultures of *E. coli* and 99.81±0.11% of the cultures of *S. aureus* were killed (see Table 1 and Figure 1).

Table 1. Antibacterial activity of the MAG OXYGEN (PLT_GEN_IONI) device following 15 minutes and 30 minutes of incubation.[a]

	E. coli	*S. aureus*
15 min	47.18±2.42	26.53±2.81
30 min	94.87±0.15	99.81±0.11

[a]Data are reported as killing percentages.

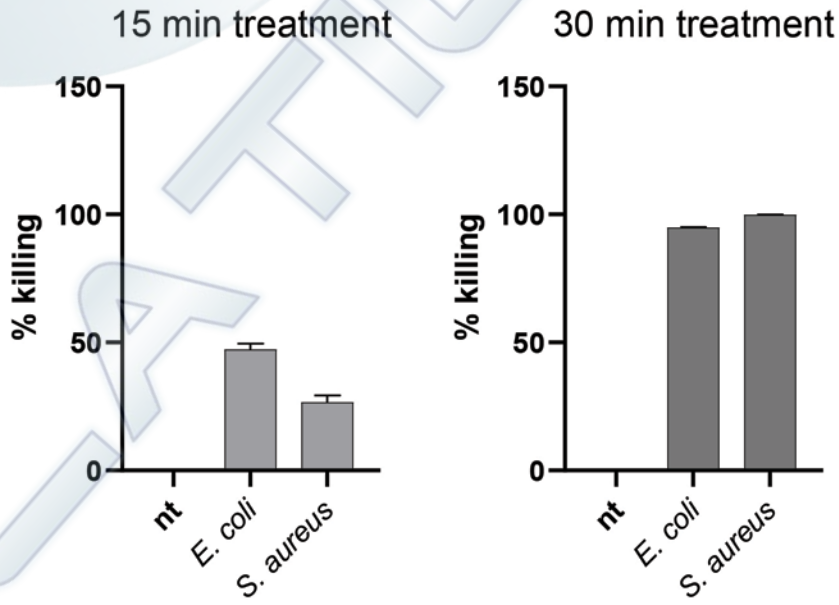

Figure 1. Graphical representation of the data reported in Table 3 in comparison with untreated samples (nt: 0% killing for the tested bacterial species). Data are reported as mean±st err of the killing percentages obtained from 3 independent experiments.

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Conclusion

Overall, the data collected indicate that the MAG OXYGEN (PLT_GEN_IONI) device had strong antibacterial effects against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. The antibacterial effects were already evident after 15 minutes treatment reaching an impressive 99% inactivation in *S. aureus* following 30 minutes of incubation.

Section 2: Antiviral activity

Preliminary experiments on the antiviral activity of the apparatus were performed using the Hazara virus (HAZV) strain JC280 (GenBank accession number M86624.1). HAZV is an enveloped negative-sense single-stranded RNA virus belonging to the *Nairoviridae* family used as a viral model of the Crimean-Congo hemorrhagic fever virus for in vivo and in vitro studies.[1] HAZV was grown in SW13 cells and titrated by immunostaining on Vero cells. Virus titer was expressed as Focus-Forming Unit per milliliter (FFU/mL).

Virus particles (10^6, 10^5, and 10^4 FFU) were diluted in 400 µL of Dulbecco's Modified Eagle's Medium (DMEM) and seeded in wells (8.87 cm^2 per well) of a 6-well plate obtaining a thin liquid layer. The plate with the virus was exposed to the modified atmosphere inside the plastic box for 15 or 30 minutes. After this time, 400 µL of DMEM were added to each virus well, thus the medium was recovered and the virus infectivity was evaluated by viral particles titration on Vero cells and expressed as FFU/mL accordance with our recently published procedure.[1] In particular, serial (1:10) dilutions of treated or untreated virus in DMEM with 2% heat-inactivated fetal bovine serum (FBSi), were used to infect Vero cells in duplicate. Twenty-four hours post infection, the cells were fixed by glacial methanol/acetone (1:1) over-night. The fixed cells were incubated with a HAZV anti-nucleoprotein antibody for 30 minutes followed by incubation with Alexa Fluor 488 anti-rabbit antibody. Finally, infected cells were counted using a fluorescent microscope. Results showed that 10^4 viral particles were completely inactivated both at 15 or 30 minutes of exposition while in the

[1] V. Monteil, C. Salata, S. Appelberg, A. Mirazimi, Hazara virus and Crimean-Congo Hemorrhagic Fever Virus show a different pattern of entry in fully-polarized Caco-2cell line. PLoS Negl Trop Dis. 2020, 14(11):e0008863. https://doi.org/10.1371/journal.pntd.0008863

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case of 10^5 viral particles the reduction of virus infectivity was 73.5% or 87.4% at 15 or 30 minutes of exposition, respectively. A sustained virus inactivation was also observed with the highest dose of virus (Table 2 and Figure 2).

Table 2. Antiviral activity of the MAG OXYGEN (PLT_GEN_IONI) device following 15 minutes and 30 minutes of HAZV incubation.[a]

	10^6 FFU HAZV	10^5 FFU HAZV	10^4 FFU HAZV
15 min	62.17±13.1	73.5±5.7	100±0
30 min	78.1±13.5	87.4±1.3	100±0

[a] Data are reported as reduction of infectivity in percentages.

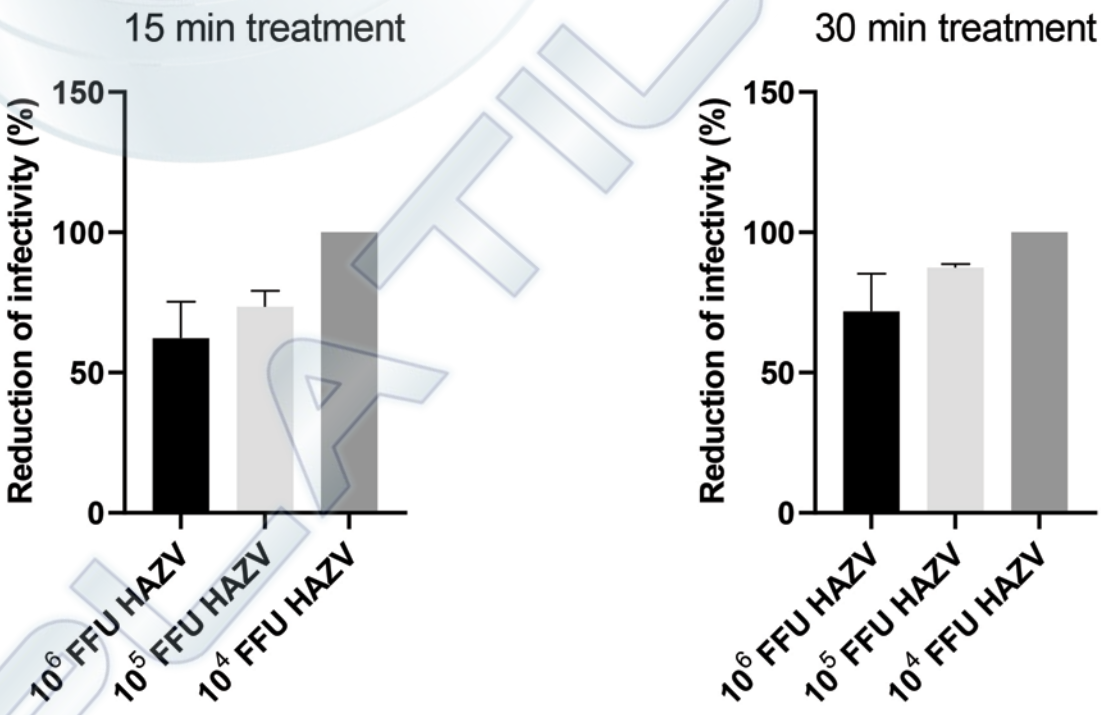

Figure 2. Graphical representation of the data in Table 1. Data are reported as percentages of mean±st dev of the infectivity reduction of HAZV, in comparison to the untreated samples, obtained from 3 independent experiments.

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Furthermore, the antiviral activity was assayed using the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Italian clinical isolate (Genbank accession number MW000351). SARS-CoV-2 is an enveloped positive-sense single-stranded RNA virus belonging to the *Coronaviridae* family, and is the causative agent of Covid-19 disease.
All studies with viable SARS-CoV-2 were performed in the certified BSL3 laboratory at the Department of Molecular Medicine of the University of Padova, Italy.

The antiviral activity of MAG OXYGEN (PLT_GEN_IONI) device against SARS-CoV-2 was tested treating 10^6, and 10^4 virus particles employing the same procedure above described for Hazara virus. After virus exposition to the modified atmosphere inside the plastic box, virus infectivity was evaluate infecting Vero E6 cells and quantifying the viral genome at 24 hours post infection by quantitative RT-real-time-PCR.
In a typical experiment, 250 µL of virus suspension was inoculated on confluent Vero-E6 cells for 1 h at 37 °C. The virus inoculum was then removed from each well and cells were overlaid with DMEM, 10% FBSi. Twenty-four hours later, cells were lysed and total RNA was extracted using the PureLink RNA Mini kit (Thermofisher), following the manufacturer's instructions, and quantified with the NanoDrop spectrophotometer (Thermofisher). Thus, a two-step RT and real-time-PCR reaction was performed according to our procedure reported in the literature[2] using specific primers targeting the RNA polymerase–encoding sequence of SARS-CoV-2 in an ABI PRISM 7000 Sequence Detection System (Thermofisher).
The quantification of viral RNA was performed using a standard curve made by six serial dilutions (from 5×10^6 to 50 copies) of a control plasmid containing the region amplified by the primers. Results are depicted in Table 3 and Figure 3.

[2] T. Pecere, F. Sarinella, C. Salata, B. Gatto, A. Bet, F. Dalla Vecchia, A. Diaspro, M. Carli, M. Palumbo, G. Palù, Involvement of p53 in specific anti-neuroectodermal tumor activity of aloe-emodin. Int. J. Cancer. 2003, 106, 836 – 847.

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Table 3. Antiviral activity of the MAG OXYGEN (PLT_GEN_IONI) device following 15 minutes and 30 minutes of SARS-CoV-2 incubation.[a]

	10^6 FFU SARS-CoV-2	10^4 FFU SARS-CoV-2
15 min	54.40±8.28	73.3±8.82
30 min	62.34±24.14	99.52±0.39

[a] Data are reported as reduction of infectivity in percentages.

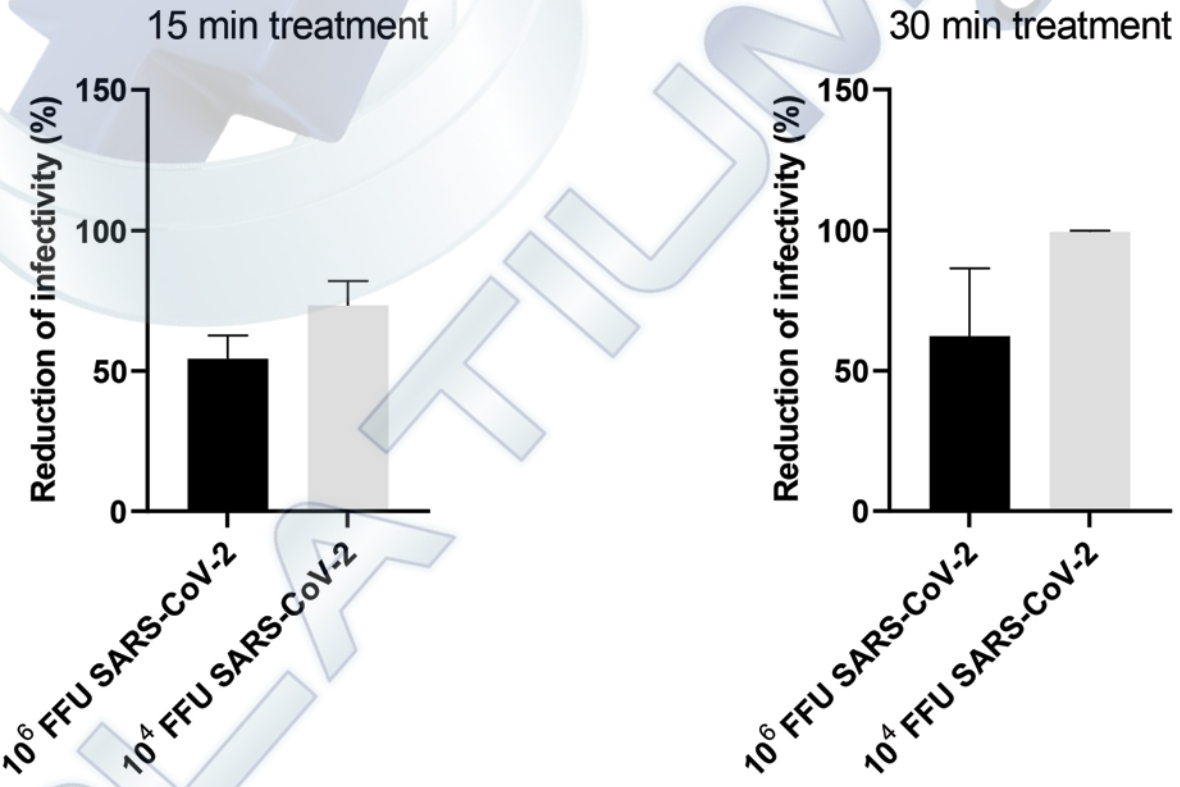

Figure 3. Graphical representation of the data in Table 2. Data are reported as percentages of mean±st dev of the infectivity reduction of SARS-CoV-2, in comparison to the untreated samples, obtained from 3 independent experiments.

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Conclusion

The data collected indicate the strong time-dependent antiviral effects of the MAG OXYGEN (PLT_GEN_IONI) device against HAZV and SARS-CoV-2 dispersed in a thin layer liquid. The antiviral efficiency is very high in the case of 10^4 virus particles that are completely inactivated (HAZV) or strongly impaired in their infectivity (SARS-CoV-2).

The antiviral effect is inversely correlated to the virus concentration and in all cases resulted higher after 30 min of exposition. It could be assumed that the antiviral activity would be even more effective in the case of virus particles in aerosol or small drops than in the thin layer liquid.

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