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Virucidal and antiviral effects of *Thymus vulgaris* essential oil on feline coronavirus

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ABSTRACT

Feline infectious peritonitis (FIP) is a fatal systemic disease of felids caused by a Coronavirus (CoV) (FIPV). In spite of its clinical relevance and impact on feline health, currently the therapeutic possibilities for treatment of FIP in cats are limited. The emergence of the pandemic Severe Respiratory Syndrome (SARS) coronavirus (CoV) type 2 (SARS-CoV-2), etiological agent of the 2019 Coronavirus Disease (COVID-19), able to infect a broad spectrum of animal species including cats, triggered the interest for the development of novel molecules with antiviral activity for treatment of CoV infections in humans and animals.

Essential oils (EOs) have raised significant attention for their antiviral properties integrating and, in some cases, replacing conventional drugs. *Thymus vulgaris* EO (TEO) has been previously shown to be effective against several RNA viruses including CoVs. In the present study the antiviral efficacy of TEO against FIPV was evaluated *in vitro*.

TEO at 27 μg/ml was able to inhibit virus replication with a significant reduction of 2 log10 $TCID_{50}$/50 μl. Moreover, virucidal activity was tested using TEO at 27 and 270 μg/ml, over the cytotoxic threshold, determining a reduction of viral titre as high as 3.25 log10 $TCID_{50}$/50 μl up to 1 h of time contact. These results open several perspectives in terms of future applications and therapeutic possibilities for coronaviruses considering that FIPV infection in cats could be a potential model for the study of antivirals against CoVs.

The pandemic 2019 Coronavirus Disease (COVID-19) caused by Severe Respiratory Syndrome (SARS) coronavirus (CoV) type 2 (SARS-CoV-2) (WHO, 2020) prompted the research on therapy and immune-prophylaxis, taking advantage of previous knowledge accumulated on SARS-CoV-1 and animal CoVs (Decaro et al., 2020). To date, no specific drug has been approved for the treatment of patients with COVID-19. However, remdesivir, an inhibitor of RNA-dependent RNA polymerase (RdRp), demonstrated promising results (Kabir et al., 2020). Moreover, studies to evaluate the efficacy of teicoplanin and monoclonal and polyclonal antibodies against SARS-CoV-2 are currently ongoing (Kabir et al., 2020).

Drugs investigated in cats for the therapy of Feline Infectious Peritonitis (FIP) have been also tested against COVID-19 in human patients (Pedersen et al., 2018, 2019).

CoVs have been long known for the FIP, a fatal systemic disease of felids. FIP virus (FIPV) is a virulent pathotype of feline enteric coronavirus (FCoV) (Kummrow et al., 2005). In spite of its impact on feline health, the therapeutic possibilities for treatment of FIP in cats are limited and effective vaccines are not available. Moreover, vaccine adverse effects have been reported (Tizard, 2020). The development of novel molecules with antiviral activity for treatment of CoV infections is now perceived as a priority in both human and animal medicine. FIPV infection in cats is considered a potential model for the study of antivirals against CoVs (Amirian and Levy, 2020).

Herbal medicinal products have sparked the interest of consumers and researchers (Hosseinzadeh et al., 2015) and essential oils (EOs) extracted from aromatic and medicinal plants have raised particular attention for their beneficial properties (de Sousa Barros et al., 2015). EOs have been reported to exhibit significant antiseptic, antibacterial, antiviral, antioxidant, anti-parasitic, antifungal and insecticidal activities (Chouhan et al., 2017; Ma and Yao, 2020). Recently, in catfish experimentally intoxicated with Thiamethoxam (TMX), *Thymus vulgaris* EO (TEO) administration partially decreased the toxic impacts of TMX (El Euony et al., 2020). EOs are also a potential reservoir of innovative

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therapeutic solutions that integrate and, in some cases, replace conventional drugs (Reichling et al., 2009). For instance, *Laurus nobilis* EO inhibited SARS CoV type 1 (SARS-CoV-1) (Loizzo et al., 2008) and a mixture of EOs was effective against the avian CoV infectious bronchitis virus (IBV) (Jackwood et al., 2010). Recently, EOs have been also shown to possess antiviral activity against SARS-CoV-2 (Asif et al., 2020). TEO has been shown to be effective against several RNA viruses including CoVs (Lelešius et al., 2019; Nadi et al., 2020).

TEO (Specchiasol Bussolengo, Verona - Italy) composition was determined in three independent experiments using the gas chromatography hyphenated with mass spectrometry (GC-MS) technique, as recently reported (Rosato et al., 2020). Details of sample preparation, instruments and GC-MS analysis methods have been previously reported (Salvagno et al., 2020; Rosato et al., 2018). Data from GC/MS analyses were expressed as area % ± Structural Equation Modeling (SEM). In all cases SEM was below 10%. Statistical analysis for SEM was performed using Microsoft Excel Office 2010 (Windows 7 Home Premium, Microsoft Corporation, USA). A total of 26 components were identified in TEO sample corresponding to 98.7% of the whole mixture. The detailed chemical composition of TEO was reported in Supplementary Table 1.

TEO at stock concentration of 928 mg/ml was initially diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA) and subsequently in Dulbecco-MEM (D-MEM).

Crandell Reese Feline Kidney (CRFK) cells were cultured in DMEM and FCoV-II strain 25/92 (Buonavoglia et al., 1995), with a titre of $10^{5.25}$ Tissue Culture Infectious Doses ($TCID_{50}$)/50 µl, was used for the experiments.

TEO cytotoxicity was assessed by XTT assay (Denizot and Lang, 1986) using the *In Vitro* Toxicology Assay Kit (Sigma–Aldrich Srl, Milan, Italy) after exposing the cells to various compound concentrations (7.25, 14.5, 29, 58, 116, 232, 464, 928, 1856 µg/ml) for 72 h. Cytotoxicity was assessed by measuring the absorbance signal (optical density, OD), spectrophotometrically. In all experiments untreated cells were used as negative control and considered at 0% cytotoxicity. Cells treated with equivalent dilutions of DMSO were used as vehicle control. After logarithmic conversion of TEO concentrations, data obtained in the cytotoxicity assays were analyzed by a non-linear curve fitting procedure. Goodness of fit was tested by non-linear regression analysis of the dose-response curve. The maximum non-cytotoxic concentration was considered as the compound concentration at which viability of treated CRFK cells decreased by no more than 20% (CC_{20}) with respect to the negative control.

The CC_{20} value of TEO was assessed at 27 µg/ml and calculated on the basis of mean ± standard deviation (SD) of three experiments. In all the experiments, DMSO did not show any effect on cells.

On the basis of the cytotoxicity assay results, the antiviral activity against the FCoV-II strain 25/92 was evaluated using TEO at 27 µg/ml and also below the cytotoxic threshold (13.5 µg/ml). The use of the substance below the cytotoxic threshold allows us to reduce toxicity and to obtain effective results at a lower cost. Confluent monolayers of CRFK cells of 24 h in 24-well plates were infected with 100 µl of FCoV-II containing 10 $TCID_{50}$, with a Multiplicity of Infection (MOI) of 0.14. After virus adsorption for 1 h at 37 °C, the inoculum was removed, cell monolayers were washed once and TEO was added. In untreated infected cells, D-MEM was used to replace the inoculum and used as virus control. After 72 h, aliquots of supernatants were collected for viral titration (Lanave et al., 2019) and RNA quantification (Gut et al., 1999).

Virucidal activity of TEO against FCoV-II was evaluated by pre-treatment of the virus (10,000 $TCID_{50}$) with TEO at 27 µg/ml and over the cytotoxic threshold (270 µg/ml) since, if used as virucide, the molecule is not posed into direct contact with the cells. In detail, 100 µl of FCoV-II were treated with TEO (1 ml) at room temperature. Virus control was used for the experiments. After 10 min, 30 min, and 1 h, aliquots of each mixture of virus-TEO and virus control were subjected to viral titration (Lanave et al., 2019).

Data from antiviral and virucidal activity assays were expressed as mean ± SD and analyzed by Analysis of Variance (ANOVA) using Tukey test as *post hoc* test (statistical significance set at 0.05).

Statistical analyses were performed with the software GraphPad Prism v.8.0.0 (GraphPad Software, San Diego, CA, USA).

Viral titres of infected CRFK cells treated with TEO and of untreated infected cells (virus control) were expressed as the log10 $TCID_{50}$/50 µl and plotted against the drug concentrations. By comparing the viral titre of untreated infected cells (4.25 log10 $TCID_{50}$/50 µl) with infected cells treated with TEO at 13.5 and 27 µg/ml, a decrease of 0.25 ($p > 0.05$) and 2.25 log10 $TCID_{50}$/50 µl ($p < 0.0001$), respectively was induced (Fig. 1A). This suggests that TEO at 27 µg/ml is able to significantly inhibit virus replication. The antiviral activity of TEO against FIPV parallels results obtained with *Thymus vulgaris* hydrosols *in vitro* against Porcine Reproductive and Respiratory Syndrome virus (PRRSV) (Kaewprom et al., 2017).

Viral nucleic acids (NAs) were expressed as log10 viral NAs/10 µl of infected cells treated with TEO and of virus control and plotted against the non-cytotoxic drug concentrations. By comparing viral load of untreated infected cells (6.53 log10 viral NAs /10 µl) with infected cells treated with TEO at 13.5 and 27 µg/ml a decrease of 0.61 ($p = 0.0005$) and 1.34 ($p < 0.0001$) log10 NAs/10 µl, respectively was observed (Fig. 1B).

The virucidal activity of TEO at different concentrations and for different contact times with FCoV-II was assessed (Fig. 2). After 10 min, TEO at 27 and 270 µg/ml determined a reduction of 1.5 ($p = 0.0008$) and 2.5 ($p < 0.0001$) log10 $TCID_{50}$/50 µl, respectively, compared to the virus control (4.25 log10 $TCID_{50}$/50 µl) (Fig. 2A). After 30 min, TEO at 27 and 270 µg/ml induced a decrease of 1.25 ($p = 0.0007$) and 3.375 ($p < 0.0001$) log10 $TCID_{50}$/50 µl, respectively, compared to the virus control (3.75 log10 $TCID_{50}$/50 µl) (Fig. 2B). After 1 h, TEO at 27 and 270 µg/ml determined a decrease of 1.25 ($p = 0.0007$) and 3.25 ($p < 0.0001$) log10 $TCID_{50}$/50 µl compared to the virus control (3.50 log10 $TCID_{50}$/50 µl). Viral inactivation occurred in a dose- and time-dependent fashion, starting from 33.33% and reaching 92.86% when TEO was used at the highest concentration (270 µg/ml), after 1 h (Fig. 2C). The virucidal activity of TEO could be explained by the ability of damaging viral envelope, thus preventing adsorption and penetration into host cells (Reichling et al., 2009) as observed by electron microscopy in herpesvirus envelope after pre-treatment with EOs (Shogan et al., 2006). Accordingly, TEO could be a valuable tool for disinfection of surfaces and it could be proposed as additive in some food preparations.

Thymus vulgaris is a Mediterranean aromatic plant, that contains EOs and lipophilic substances (Nabavi et al., 2015) and its extracts are rich in thymol, carvacrol, p-cymene, and γ-terpinene (Kowalczyk et al., 2020).

Thymus vulgaris has demonstrated antiviral activity against herpes simplex virus (HSV) (Nolkemper et al., 2006), influenzavirus (Vimalanathan and Hudson, 2014), Newcastle Disease virus (Rezatofighi et al., 2014), PRRSV (Kaewprom et al., 2017) and IBV (Lelešius et al., 2019), even if the antiviral mechanism has yet to be clarified. Conversely, the inhibition of replication of Human Immunodeficiency Virus *in vitro* by TEO was elucidated (Feriotto et al., 2018).

The chemical composition of TEO revealed the presence of 26 distinct molecules, the main fractions of which were thymol, p-cymene, γ-terpinene, β-linalool, caryophyllene. In order to reduce the cytotoxicity of TEO, it would be interesting to identify the active molecules and to test them individually. As expected, the major component of the TEO used in this study was represented by thymol. Thymol fraction has proved to have efficacy against HSV (Sharifi-Rad et al., 2017) and influenzavirus (Alburn et al., 1972). Other less represented fractions of TEO should be tested to assess their antiviral activity.

In conclusion, we demonstrated the *in vitro* antiviral and virucidal effect of TEO against FCoV in CrFK cells. These studies open several perspectives in terms of future applications and therapeutic possibilities for human and animal coronaviruses.

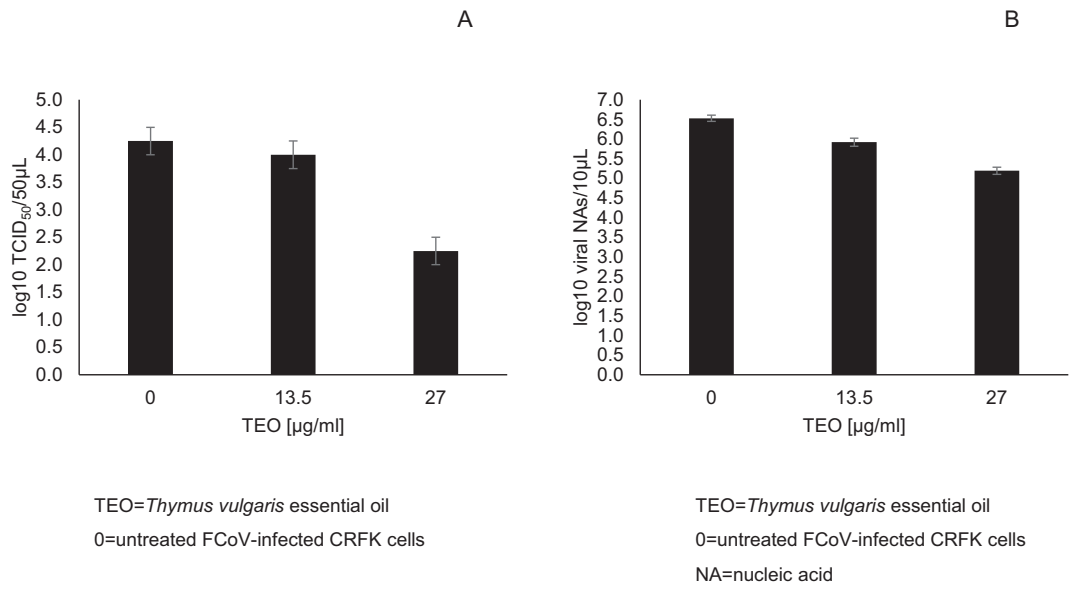

Fig. 1. Viral titres of the supernatants collected at 72 h post infection from FCoV (10 $TCID_{50}$)-infected CRFK cells untreated and treated with *Thymus vulgaris* essential oil (TEO) at different concentrations (13.5 and 27 µg/ml).
Viral titres were evaluated by endpoint dilution method, expressed as log10 $TCID_{50}$/50 µl and plotted against TEO at different concentrations (A). Viral nucleic acids were quantified by qPCR, expressed as log10 viral NAs /10 µl and plotted against TEO at different concentrations (B). Bars in the figures indicate the means. Error bars indicate the standard deviation.

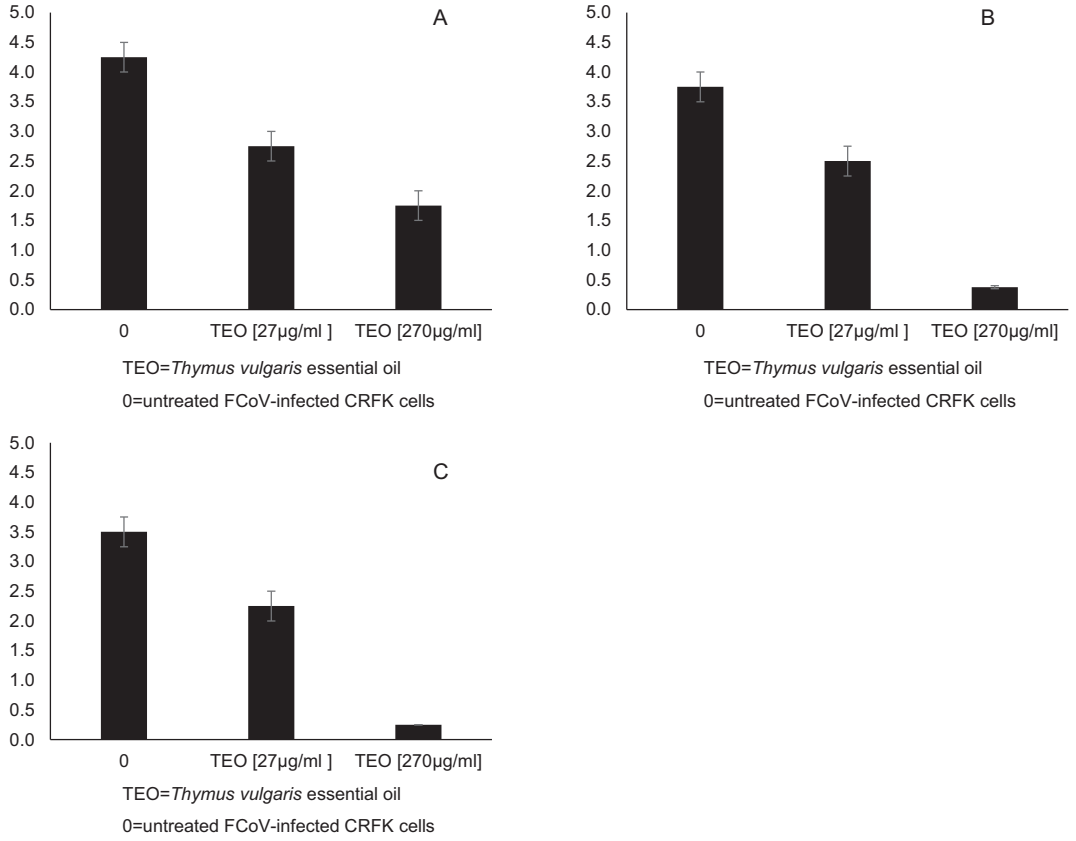

Fig. 2. Virucidal effect of TEO at different concentrations (27 and 270 µg/ml) against FCoV (10,000 $TCID_{50}$). The virus was incubated with TEO for 10 min (A), 30 min (B) and 60 min (C) at room temperature and subsequently titrated in CRFK cells. Viral titres of FCoV were expressed as log10 $TCID_{50}$/50 µl and plotted against TEO at different concentrations. Bars in the figures indicate the means. Error bars indicate the standard deviation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2021.04.024.

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