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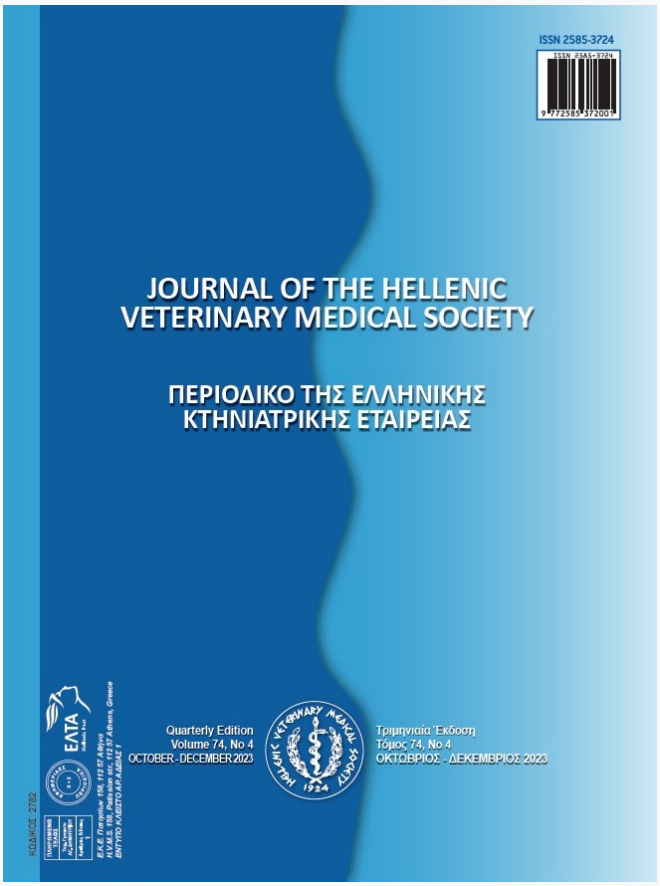

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Research article
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In vitro evaluation of a natural food supplement as inhibitors of feline herpesvirus replication

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ABSTRACT: The feline herpesvirus (FHV) is a widely diffused and highly contagious virus that represents a common health problem in cats. It is frequently associated with diseases of different pathogenicity that can be particularly severe in young kittens leading to viral pneumonia and sometimes death. Unfortunately, there isn't an effective therapeutic protocol against the virus. Several studies concerning the application of alternative treatments against herpesviruses have been performed with promising results, in both human and veterinary medicine. The present study aims to investigate the *in vitro* antiviral properties against FHV of a commercially available food supplement HELP-TH1 (Camon, S.p.A., Italy) against FHV. The HELP-TH1 is principally composed of *Ganoderma lucidum, Cordyceps sinensis,*and *Trametes versicolor*. Since those mushrooms have been largely used in traditional medicine for different purposes and several studies indicate their antiviral properties when used alone, we tested if their properties are maintained when acting as a synergy. For such a reason, we tested the possible antiviral properties of HELP-TH1, a commercially available food supplement against FHV. The role of HELP-TH1 was evaluated by the plaque reduction assay and real time PCR. These data indicated that, in the *in vitro* experimental conditions, HELP-TH1can reduce cytopathic effect of the virus and its relativethe viral load demonstrating its antiviral properties.

Keywords: Feline herpesvirus; phytotherapy; antiviral activity

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INTRODUCTION

Feline herpesvirus 1 (FHV-1) is a double-stranded DNA virus belonging to the family *Herpesviridae*, genus *Varicellovirus*. FHV-1 infects several members of the *Felidae* species, but domestic cats are the main hosts.FHV-1 is a highly contagious virus that represents a common health problem in catswith prevalence ranging from 5 to 70 % (Binns et al., 2000; Mochizuki et al., 2000; Helps et al., 2005;Lickey et al., 2005; Low et al., 2007; Blanco et al., 2009;Zicola et al., 2009; Nguyen et al., 2019;). A feature of herpesvirus is the presence of a life-long latent infection.It seems that 45% of latently infected cats can shed the virus spontaneously or, much more frequently, following a stressful event (Gaskell and Povey, 1977; Gaskell et al., 1985; Thiry et al., 2009; Gould, 2011). The latently infected cats, that shed the virus without clinical evidence, represent a silent reservoir of virus and play a key role in the epidemiology of the disease enabling the maintenance and spread of infection.

Following the infection, the virus replicates in the cells of conjunctiva, cornea, upper respiratory tract, and neurons and it is frequently associated with diseases of different pathogenicity that can be particularly severe in young kittens leading to viral pneumonia and sometimes death (Gaskell et al., 2007; Thiry et a., 2009; Mazzei et al., 2019). Both modified-live and inactivated virus vaccines are available for to the prevention of disease. Although both vaccine result in less severe clinical signs and reduced viral shedding, mild clinical signs may be still present after vaccination (Gaskell et al., 2007; Thiry et a., 2009; Summers et al.,2017).

Unfortunately, there isn't a well-defined therapeutic protocol against the virus and so far, the available drugs are virostatic that, despite being able to reduce the replication of the virus and the severity of clinical manifestations, must be administered frequently and are not completely curative (Thomasy and Maggs, 2016).

In recent years, various herbal medicines and phytotherapeutic compounds, obtained from medicinal plants and fungi, have indicated promising results for the treatment of infectious disease owing to their antipyretic, anti-inflammatory, and antiviral natural properties (Teplyakova and Kosogova, 2015; Elkhateeb et al., 2019; Shahzad et al., 2020). Several studies concerning the application of alternative treatments against herpes viruses have been performed with promising results, in both human and veterinary medicine (Chattopadhyay et al., 2010;Astani et al., 2011; Teplyakova and Kosogova, 2015). *Ganoderma lucidum* (Basidiomycota: Ganodermataceae) is aspecies of basidiomycetes that has been known as the mushroom of immortality in Chinese and Japanese traditional medicine and has long been used in oriental folk medicine for the prevention and treatment of various kinds of diseases (Sanodiya et al., 2009). It hasmany biologically active components with a powerful immuno-modulating activity and antitumoral and antiviral properties (Chien et al., 2004; Liu et al., 2004; Akbar et al., 2011; Wang et al., 2012; Yan et al., 2014; Zhao et al., 2018). In particular, several neutral and acidic protein-bound polysaccharides (NPBP and APBP) and a proteoglycan (Ganoderma lucidum proteoglycan, GLPG) have indicated *in vitro* inhibitory activities against herpes simplex virus 1 and 2 (Eo et al., 1999; Eo et al., 2000; Kim et al., 2000; Oh et al., 2000; Teplyakova and Kosogova, 2015; Elkhateeb et al; 2019).

Cordyceps sinensis is a well-known mushroom of traditional Chinese medicine characterized by severaltherapeutic properties (Holliday et al., 2008; Yue et al., 2013; Yan et al., 2014). Recently, it has become increasingly important in the scientific communities due to its many bioactive constituents and their therapeutic actions against some nervous, cardiovascular, respiratory, renal, and hepatic diseases, their immunomodulatory and anti-inflammatory effects, and their antioxidant properties. Moreover, several bioactive compounds isolated from *Cordyceps sinensis* were studied for their antitumoral and antiviral activities (Chen et al., 2006; Tian, 2011; Wang et al., 2012; Zhu et al., 2016; Saleh et al., 2017).

Trametes versicolor, or *Coriolus versicolor*, is an ancient Chinese medicinal mushroom widely known as an important source of immunomodulatory compounds (Cruz et al., 2016).

Polysaccharides, polysaccharopeptide (PSP), and polysaccharopeptide Krestin (PSK) are the most important bioactive components of *T. versicolor* stimulating both humoral and cell-mediated immune responses (Ho et al. 2004; Lee et al. 2006; Li et al., 2011; Jedrzejewski et al. 2015; Cruz et al., 2016; Jedrzejewski et al. 2019). Moreover, *Trametesversicolor*'s polysaccharides are studied for their medicinal value in cancer therapy and their antiviral and antimicrobial role, indicating a positive role in both *in vivo* and *in vitro* trials (Standish et al., 2008; Ferreira et al., 2010; Ozgor et al., 2016; Habtemariam, 2020).

PSP is known to have antiviral properties and seems to be effective against herpes simplex virus (HSV), influenza virus, Bovine herpes virus1(BoHV-1) (Krupodorova et al., 2014; Manoj et al., 2017).

In this study, we investigate the *in vitro* antiviral effects of HELP-TH1 (Camon S.p.A, Italy), a newly commercially available feline food supplement, against FHV-1. HELP-TH1 is commercialized as a food supplement aimed to stimulate feline immune responses, by enhancing Th1 lymphocytes. Interestingly it is composed by several natural herbal and mushroom extracts that are known in traditional medicine. Among others *Ganoderma lucidum, Cordyceps sinensis, Trametes versicolor* are those mostly represented in the compound. Since those have been already singularly tested for their antiviral and antimicrobial activities, the purpose of this study was to investigate whetherin the commercially available food supplement HELP-TH1 (Camon S.p.A, Italy), those natural products could have any *in vitro* antiviral properties against FHV-1 by acting as a synergy.

MATERIALS AND METHODS

Cells and viruses

Crandell-Rees Feline Kidney (CRFK) cells were grown at 37ºC in 5% CO_2 in Medium Essential Medium (MEM) (Gibco, USA) supplemented with 10% fetal calf serum (Euroclone S.p.A., Italy), 1% Gentamycin and 1% Penicillin/Streptomycin (Corning, USA).

Feline Herpes Virus-1 (FHV-1) adapted to CRFK cells was purchased from ATCC (FHV-1 VR-636™). The virus was grown in CRFK cells and quantified and titrated by plaque assay.

Origin and preparation of the compound

HELP-TH1 is a commercially available food supplement (Camon S.p.A, Italy) that is principally composed of *Ganoderma lucidum, Cordyceps sinensis,* and *Trametes versicolor* (5% each) with a grade of purity ≥ 95%. The compound (Lot. No20M291) was weighted and 10 mg were resuspended in 1 ml of MEM, the liquid mixture was incubated for 4h at 20 ±2ºC under constant shaking and it was finally serially diluted in MEM (10 dilution points from 1:2 to 1:1024).

Cytotoxicity assay

To monitor the possible cytotoxic effect of HELP-TH1 on CRFK cells, their viability was measured by the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, USA) following manufacturer's instructions as also described in Forzanand colleagues (Forzan et al., 2022). In detail, CRFK cells were plated in a 96-well microplate at a concentration of $1x10^4$ cells/well and incubated for 24hrs at 37°C in 5% CO_2 incubator. Cells were washed twice in serum-free MEM without phenol red, then incubated for 2h at 37°C with serial dilutions of HELP-TH1 (1:2 to 1:1024;from 5 mg/ml to 9,75 ng/ml). After 1 h and 30 min, 10 µl of lysis buffer from the kit was added to the high control group (positive control of the kit) and the plate was incubated for additional 30 min in the same conditions as before. At the end of 2 hrs incubation, 100 µl of the Working Solution was added to each well. The plate was protected from light and incubate it at the room temperature for 30 min.- Optical density was determined on an ELISA plate reader (Multiskan FC, Thermo Scientific, USA) at absorbance 490nm. Cytotoxicity percentage was measured following manufacturer instruction using the equation: Cytotoxicity (%) = Test Substance - Low Control/High Control - Low Control x 100. The test has been conducted in triplicate and therefore, calculation of the percentage of cytotoxicity was made using the mean value of each test.

Plaque Reduction Assay

The assay was performed on CRFK cells that were infected with different titres of FHV-1 that was pre-treated or not with HELP-TH1. The supposed antiviral activity was then measured by counting the number of plaques in the two treatments. In brief, CRFK cells were plated at $1x10^5$/well in a 24 well tissue culture plate and grown for 16 hrs. The virus was serially diluted in serum-free MEM from 10^{-2} to 10^{-5} and 100 µl of each dilution corresponding to $6.5x10^2$ to $6,5x10^{-11}$ plaque forming unit (PFU), respectively were used for CRFK cells infection, a sinfected control group. Same volumes and dilution of FHV-1 as in the infection control group were pre-incubated for 1hr at 20 ±2ºC with different concentrations of the compound from 1:256 to 1:1024(39; 19,5 and 9,75 ng/ml final concentration) for the treated experimental group. After pre-incubation cells were infected for 1hr at 37°C, 5%CO_2. After 1hr the cells were washed twice in serum free medium and 500µl of 2% low melting agarose (Merck KGaA,Germany)were added to each well and allowed to coolfor 30 min at20 ±2ºC. Finally, 500µl of MEM, and 10% FCS was (Gibco, USA) were added to each well. Infection was carried

out for 72h and the cytopathic effects were monitored daily by microscopy. At the end of the experiment, cells were fixed for 1hr at 20 ±2ºC with 20% formaldehyde (Merck KGaA, Germany). Agarose was removed from each well by a needle and the cells incubated for 15 min with a solution of 0.5% crystal violet dye. The dye was staining solution (Merck KGaA,Germany). The staining solution was finally removed, and the plaques were observed by microscopy. Plaques were counted by dividing each well in 12 squares by a marker and plaques counted by eye and then, in order to better discriminate between a real plaque and a possible defect of the staining or a scratch in the cell monolayer, by visualisation on the microscope and. Every well was counted for plaques at least three times.

Evaluation of antiviral activity by real-time PCR

The real-time PCR was performed in parallel with the plaque reduction assay. The antiviral effect of the product was evaluated by quantifying and comparing the viral load of infected controls with the treated experimental samples. In order to evaluate the antiviral potential of the compound to constraint viral replication, at 72h post infection (p.i.), cells and supernatants were harvested and DNA was extracted by DNeasy blood and tissue mini kit (QIAGEN, Germany). The DNA was quantified by nanodrop (Thermofisher, USA) and used as template in a duplex qPCR assay with TaqMan method. The qPCR assay was designed using published and validated primer-probe combination designed for FHV-1 quantification (Lee et al., 2019) and for feline RPP30 gene was used as control gene (Ertl et al., 2014).

Primers and real-time probes were synthesized by Eurofins Genomics (Germany). Probes were synthesized using the fluorophore FAM (FHV-1 detection) and HEX (RPP30 gene control detection).

The experiment was carried out usingthe iTaq Universal Probe Supermix kit (Biorad, USA). Real time assays were conducted in duplicate in Rotor-gene Real-time thermal cycler (Corbett, Australia). To validate the real-time assay, preliminary tests of system efficiency and specificity were performed in duplex qPCR on 5 scalar dilutions of CRFK cellular and FHV-1 viral DNA (efficiency 99%, specificity 100%). For the calculation of the antiviral effect the $2^{-\Delta\Delta CT}$ method was used (Pfaffl et al., 2002), the differences in the amount of viral load between treated and control groups were then analysed. $A2^{-\Delta\Delta CT}$ value indicates the over-expression (>1) or under-expression (<1) of the viral genome in the treated group compared with the control group.

RESULTS

Cytotoxicity Assay

The results obtained from the cytotoxicity assay indicated that from the concentration of 39 ng/ml (1:256 dilution) HELP-TH1 did not show any toxic effect on CRFK cells indicating its tolerability by cell. Therefore, the antiviral effect of the compound was evaluated starting from that dilution.

Plaque reduction assay

The results indicated at least a200-fold reduction in viral titer in the presence of HELP-TH1at all the 4 viral dilutions (from 10^{2} to 10^{-5}). There was no evidence of a dose-dependent effect of the product since the antiviral effect does not vary in relation to the concentration of the tested product (1:256; 1:512; 1:1024). Pictures taken prior to plaque staining indicated the reduction of viral cytopathic effect in the presence of all HELP-TH1 concentrations (Fig.1).

Reduction of viral load monitored by qPCR

In the molecular assay, the HELP-TH1 has shown antiviral properties in all tested concentrations (1:256, 1:512, 1:1024) at different titers of viral inoculum, as indicated by the $2^{-\Delta\Delta CT}<1$ value (Table 1).

The real time assay on the concentrations of 10^{-4} and 10^{-5} of FHV-1 did not result in any detectable signal, therefore no $2^{-\Delta\Delta CT}$ was calculated.

Table 1. Result of $2^{-\Delta\Delta Ct}$ indicating a reduction of viral replication dose depending

FHV-1	HELP-TH1 1:256	HELP-TH1 1:512	HELP-TH1 1:1024
10^{-2}	0.04	0.09	0.2
10^{-3}	0.01	0.01	0.07

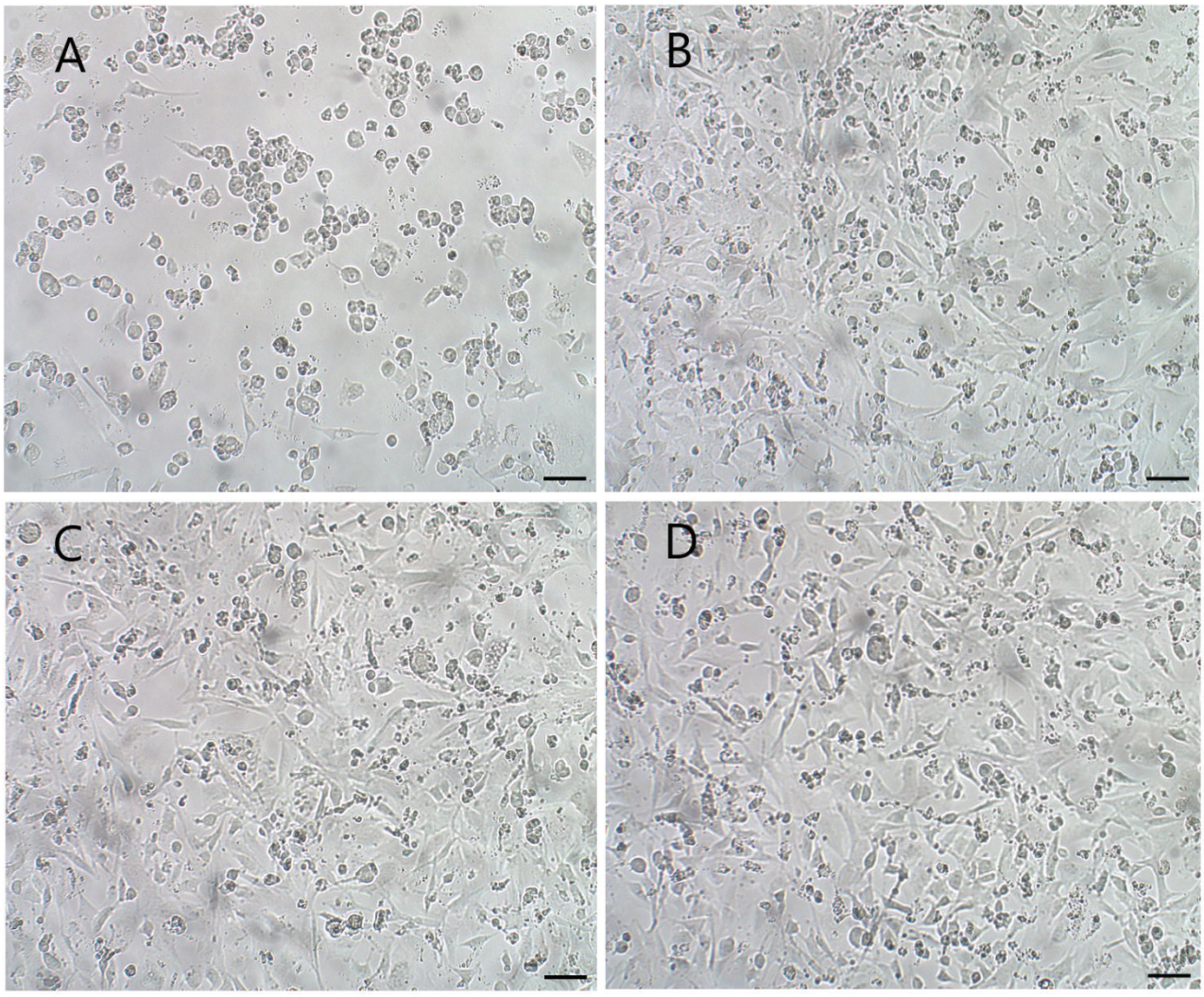

Figure1. Imagine of CRFK cells infected with FHV only (A) or with 39; 19,5; and 9,75 ng/mlof HELP-TH1 (B,C,D respectively). Scale bar is 100 nm.

DISCUSSION

Due to the little impact on the environment and the limited side effects and toxicity, the use of natural compounds as an alternative to synthetic drugs has recently increased. Furthermore, a therapeutic protocol based on treatments with natural substances could also be considered as a valid solution for the antibiotic resistance issue. An increasing number of plants and fungi have been tested for their therapeutic properties and several compounds have been isolated from these natural sources including flavonoids, alkaloids, terpenes, polysaccharides, steroids, and phenolic acids that have been tested either alone or in combination. Although most of those natural remedies derive from Traditional Chinese Medicine and have also been largely used in several developing countries, nowadays these natural remedies are used all over the world. Herbal medicines have been tested against several viral diseases of both farm animals and pets (Yasmin et la., 2020). The main aim of those studies was to find a valid alternative to traditional therapies owing to the emergence of resistance against traditional antivirals (Murayama et al., 2006; Buhner, 2021; Tahmasbi et al., 2021). Most of those investigations have been performed *in vitro* on cell lines, achieving interesting results. In this paper, we tested the antiviral properties of the commercially available feline food supplement HELP-TH1 (Camon S.P.A., Italy) againstFHV-1.Although HELP-TH1 is commercialised as a food supplement improving the immunological state of felines, we were more interested in testing the ability of the compound to act against viral replication. This consideration has been made knowing part of the composition of the supplement, since it mostly contains mushroom extracts that have been previously tested for their activity. Although

natural compounds are considered to have minimum toxicity, it was essential to test this on cells lines prior any other experiment. Our results on CRFK cell lines indicated that HELP-TH1 could be used at a starting concentration of 39 ng/ml, since on higher concentrations has resulted as toxic. From the experimental tests, the food supplement has demonstrated an *in vitro* antiviral efficacy on CRFK cells infected with FHV-1. The plaque reduction test has indicated a clear inhibition of the cytopathic effect of the virus when incubated with HELP-TH1 independently from the concentration used. The reduced viral replication was further confirmed by real time PCR, showing an evident decrease of viral load indicated by the reduction of the presence of the viral gene between 5 ($2^{-\Delta\Delta CT}$= 0.2) and 100 times ($2^{-\Delta\Delta CT}$= 0.01) compared to the control group. In the molecular assay, it was possible to show a variation in HELP-TH1 effect in relation to the concentration, being 1:256 the most effective concentration against both 10^{-2} and 10^{-3} virus dilution.

Interestingly, we noticed a different effect of HELP-TH1 depending on the test used to monitor its antiviral efficiency. By plaque assay, the reduction of CPE was effective but independent on the dose used, since the same effect was detectable at 39; 19.5; and 9.75 ng/ml. On the other hand, by real time PCR, the most effective reduction of FHV-1 genome was detected by using 39 ng/ml. Recently, two independent reviews summarized the research conducted on whole plant extracts against HSV-1 and/or HSV-2 *in vitro* and *in vivo*, describing the chemical components and the mode of action against Herpes Simplex replication (Garber et al., 2021; van de Sand et al., 2021). As some plant extracts act by inhibiting gD of gB binding to the host cells, other scan inhibit replication by acting against the viral Timidine Kinase (TK). Those reviews indicate that different botanicals, by their various active constituents, have various mechanisms of action, by directly inactivating virions or by targeting viral attachment, penetration, DNA replication, or even gene expression. Since HELP-TH1 is a synergy of several plant extracts, we canspeculate that the food supplement could act against FHV-1 replication on different levels. By pre-incubating HELP-TH1 with FHV-1 it is possible that viral binding and entry has been partially inhibited even using concentration such as 9.75 ng/ml, which could be explained by the absence of CPE detected by plaque assay. On the other hand, at the same concentration, it was not possible to see a clear effect of the compound of viral replication monitored by real-time PCR. Since the reduction of viral DNA was detected only when using 39 ng/ml it is possible that a different chemical compound present in HELP-TH1 could act against viral TK but only when a higher concentration is used. Our results support what is already present in literature regarding three main natural extracts present in HELP-TH1. Furthermore, our study could represent an innovation regarding the possible use of different natural products as a synergy. This approach could be adopted by future studies since, at least against viruses, different natural extract could act against different steps of viral replication (e.g. attachment and entry). Of course, this could each time being different depending of viruses tested. Interestingly it appears that the supplement, at least against FHV-1, could have a dual effect, enhancing the immunological state of the animal and by establishing and antiviral state.

CONCLUSIONS

At the state of the study, we are not in the position to understand which active compound is mainly active against FHV replication, nor whether the synergy has a stronger effect than each single plant extract. Further studies need to be performed to address these and other queries. In conclusion, our data suggest that HELP-TH1 can be considered as a valid food supplement to reduceFHV-1 replication when tested on an *in vitro* system.

CONFLICT OF INTEREST

None declared.

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