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Antiviral effect of *Hornstedtia bella* Škorničk essential oil from the whole plant against vaccinia virus (VV)

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ABSTRACT

In the prevention of epidemic and pandemic emerging and neglected viral infections, natural products are an important source of lead compounds. *Hornstedtia bella* Škorničkis is a rhizomatous herb growing in the forest of central Vietnam. *Hornstedtia bella* essential oil (Hb EO) was recently characterised by our group as endowed of antimicrobial activity against *Staphylococcus aureus* Methicillin-Resistant strains. Here, we describe for the first time the evaluation of Hb EO against a spectrum of viruses responsible for important human diseases. Hb EO resulted active against Vaccinia virus (VV) (EC_{50} values 80 μg/mL), closely related to variola virus, causative agent of smallpox. Hb EO was able to strongly reduce the viral VV titer in cell-based assay at not cytotoxic concentration and its potential mode of action was characterised by virucidal activity evaluation followed by time-of-addition assay. Furthermore, Hb EO antiviral activity was implemented in a combination study with the mycophenolic acid.

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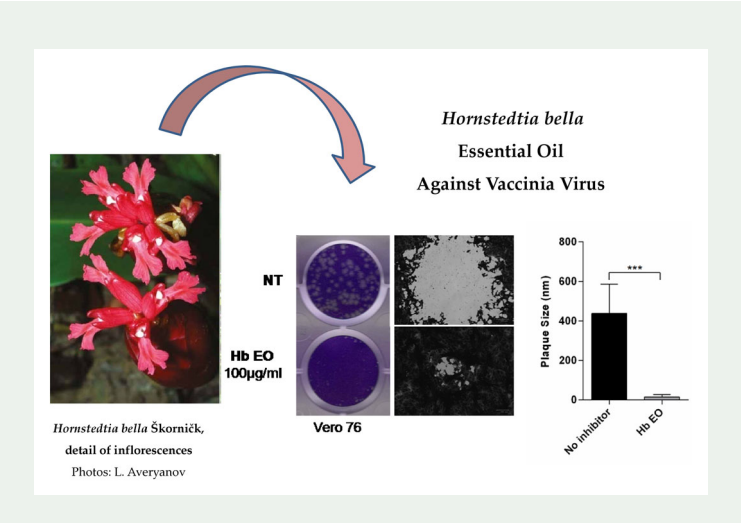

1. Introduction

Despite the impressive progress carried out in the field of biomedical sciences, in the last decades the incidence of emerging and neglected lethal viral infections mainly belonging to the Filoviridae, Arenaviridae, Bunyaviridae, Paramyxoviridae and Coronaviridae families have considerably impaired human health (Parvez and Parveen 2017). The worldwide vaccination campaign at the end of the 1970s determined the eradication of smallpox. However, the growing number of cases of diseases linked to orthopoxvirus diseases (Shchelkunov 2013) as the zoonosis caused by monkeypox in the United States (Sklenovská and Van Ranst 2018), the presence of endemic disease in Central and West Africa, increased the need for knowledge of these viral pathogens. Moreover, human vaccinia infections resulted recurrent in Brazil, Colombia (Oliveira et al. 2017; Styczynski et al. 2019) and the Indian continent. In part because of the concern that variola could be used as an agent of bioterrorism, it is important to find anti-pox agents that could be employed in case of smallpox outbreaks and complications following the potential re-introduction of live VV vaccines. In the context of prevention and treatment of epidemic and pandemic emerging and re-emerging viral diseases, products of natural source could have great medicinal value.

In recent years, natural products have been extensively used as a source of chemical drug leads in a multidisciplinary program of modern drug discovery against clinical diseases. Most drugs that are broadly used nowadays derive from herbs or other products of natural origin (Shen 2015; Redeploying plant 2020), and thus they will continue to be raw materials essential for the development of new therapeutic agents (Cragg and Newman 2013).

Hornstedtia bella Škorničk is a large terrestrial rhizomatous herb that has been recently identified (Leong-Škorničková et al. 2016) and collected from central Vietnam. No uses were reported for this plant, but indigenous knowledge refers that inflorescences and young leafy shoots are used for the preparation of soups and various

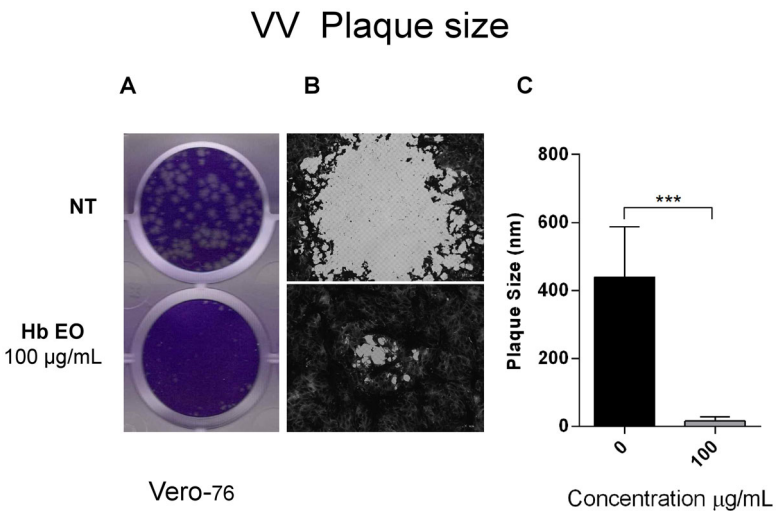

Figure 1. Plaque size reduction by Hb EO. Effect of Hb EO on the area of plaques formed by the VV in infected Vero-76 cells (A) 20 X (B) The histograms show the size of plaque area of Hb EO treated wells compared to that of not treated wells. Pictures were taken 72 h post-infection using ZOE Fluorescent cell imager (Bio-Rad) (bar size = 100µm). Areas of infection were measured using ImageJ software on images randomly acquired from separated experiments (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, $n = 3$ at least).

dishes. Its essential oil has been from us lately described as endowed of interesting antimicrobial activity against *S. aureus* MRSA and *Candida* strains (Donadu et al. 2020).

Here, we report the evaluation of *Hornstedtia bella* essential oil (Hb EO) against a broad spectrum of viruses. In our assays, Hb EO resulted to be significantly able to reduce VV titer and, to our knowledge, there is no documented information about the inhibitory effect of Hb EO on this virus. Moreover, Hb EO potential mode of action was investigated by virucidal assay and in time of addition. Furthermore, the safety profile of Hb EO was analyzed through Transepithelial electrical resistance (TEER) experiment, and additionally, we showed that a combination of Hb EO with mycophenolic acid (MA) increased the inhibitory effect of MA.

2. Results and discussion

Within a project aimed to investigate the potential biological activity of essential oils from newly identified plants (Chaves-López et al. 2018; Trong Le et al. 2020a, 2020b) all over the world, we here describe the anti-VV activity of Hb EO. *Hornstedtia bella* Škorničk is a recently identified herb that grows spontaneously in central. The strongest inhibition of the Hb EO against *S. aureus*, MRSA, *S. epidermidis, C. tropicalis and C. parapsilosis* has been recently described by us as well as the EO also demonstrated inhibition against *T. vaginalis* (Donadu et al. 2020). Given the known antiviral activity of terpenes against several viruses (Wen et al. 2007; Astani et al. 2010, 2011) and their mechanism of action, as mainly described as a result of direct interaction with free virus particles (De Logu et al. 2000; Brand et al. 2016; Camero et al. 2019), we have further analyzed the chemical composition of Hb EO to confirm its percentage

composition of terpenes: monoterpenes, oxygenated monoterpenes (as α-pinene, β-pinene, 1,8 cineole) and sesquiterpenes. To this end, in addition to the gas chromatography and mass spectrometry the chemical structures have been confirmed by nuclear magnetic resonance (1H NMR and 13 C NMR). Anyhow different targets of several essential oils and components are described by many authors (Armaka et al. 1999, Pusztai et al. 2008; Swamy et al. 2016; Boukhatem M. 2020). Burke et al. (2004) describe the essential oil of Australian lemon myrtle (*Backhousia citriodora*) as moderately efficacious and safe in the treatment of infection caused by molluscum contagiosum virus (MCV) in children (Burke et al. 2004). MCV is a worldwide cutaneous infection caused by a large DNA virus which as VV belongs to Poxviridae family and that affects both children and adults. This paper reports for the first time the selective anti-VV activity of Hb EO is reported for the first time. As shown in Tables S1 and S2, the Hb EO (from the whole plant) EC_{50} value was 80 μg/mL whereas the CC_{50} was >300 μg/mL in Vero-76 cells, indicating that the antiviral effect was not related to cytotoxicity. Interestingly, after 3 d of incubation, the inhibition of plaque formation compared to not treated control wells was also associated with plaque size reduction. The plaque number or size reduction and histogram of the plaque size are shown in Figure 1. Measurements of the surface area of all plaques photographed were made using Image J software. Without treatment, VV plaques were generally larger than VV plaque in Hb EO treated wells. Indeed, the Hb EO resulted completely ineffective against other tested viruses HIV-1, YFV, BVDV, Sb-1, CV-B4, hRSV A2, Reo-1, HSV-1, with EC_{50} values over 100 μg/mL demonstrating its specificity against VV. Accordingly, to obtain a detailed insight into the efficacy of Hb EO against VV, a yield reduction assay was performed (Figure S2). Treatment with non-cytotoxic concentration (100 μg/mL) caused a significant reduction of viral titer ($P < 0.05$, Mann-Whitney test, almost 3 logs). To explore whether the Hb EO directly inactivates VV particles, a virucidal assay was performed with a not cytotoxic concentration of EO. This treatment did not produce a significant reduction of VV infectivity demonstrating that EO is not endowed of direct virucidal activity (data not shown). Therefore, to find out the inhibitory step(s) in the VV life cycle targeted by Hb EO, we performed a Time of addition assay (Figure S3). Since some essential oils and also plant extracts are known to interfere with the cell surface protein (Ehrhardt et al. 2007; Pourghanbari et al. 2016) directly masking virus receptors, Hb EO was tested in a pre-treatment assay. Obtained results showed that Hb EO added 2 h before virus infection and then removed before infection did not exert any inhibitory activity. Inhibition was instead observed when the Hb EO was added during the infection period. Its inhibitory effect is then exerted at an early step of the viral cycle. A comparable titer reduction is evident also when EO was added 8 h post infection. Considering that in VV life cycle virion assembly is initiated at 6 h post-infection (Tolonen et al. 2001), this decrease in viral titer could be associated with cell to cell spread of the virus. We can hypothesise that the spread is reduced in the presence of Hb EO and this decline is also supported by the reduction of plaque diameter (Figure 1). Since the beginning of medicine, a precious contribution to the management of illness derived from Combination Therapies. The mixture of natural products and plants is a common practice in Chinese traditional medicines (Wang et al. 2017). Combining compounds with additive or synergistic antiviral effects is an

established approach to enhance antiviral potency as well as reduce potential toxicity and damaging effects. For example, Pourghanbari and coauthors demonstrated that a combination of *Melissa officinalis* essential oil with oseltamivir increased the inhibitory effect of oseltamivir (Pourghanbari et al. 2016). In this study, we used two-drug combination experiments to evaluate the *in vitro* efficacy of Hb EO combined with MA, an available drug known as a potent inhibitor of VV virus in cell culture. MA inhibits cellular inosine monophosphate dehydrogenase. The final result of this process is the inhibition of viral and cellular RNA, DNA, and protein synthesis (Smee et al. 2001). Considering that Time of Addition results suggest the involvement of Hb EO in an early phase of inhibition, these products have distinct mechanisms of action and then with their combination, we can increase antiviral activity as well as to minimise the induction of potential drug resistance. As showed in Figure S4, the evaluated combinations of these two products determine both an improvement of antiviral activity and protection of monolayers from VV infection. The safety profile of Hb EO was also investigated on epithelial cell monolayer of Caco-2 cells. Hb EO evaluated at not cytotoxic concentration of 100 μg/mL did not affect the TEER and then the integrity of monolayer until 48 h post-treatment (Figure S5). Therefore, it may be suggested as a suitable candidate for future applications and studies. Finally, starting from these findings, we decided to check if the antiviral activity of Hb EO from the whole plant has been kept also in leave and rhizomes essential oils. Among these two oils (Table S3) only rhizomes showed a similar spectrum of activity with an EC_{50} value of 56.5 μg/mL while EO from leaves resulted cytotoxic.

3. Conclusions

In conclusion, Hb EO was here shown to be active against Vaccinia virus inhibiting a step of its replicative cycle that occurs during the early phase of viral infection and probably during entry. Further studies are required to clarify more precisely the mechanism of antiviral action. However, additional research is ongoing to investigate whether Hb EO can exert antiviral properties as a result of the complex interactions between their constituents or being associated with their main components.

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Disclosure statement

The authors declare that they have no conflicts of interest.

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Author contributions

Conceptualization, D.U., P.C., G.S., and M.G.D.; Data curation, G.S., G.Se., S.M.; Formal analysis, G.S, D.U., H.T.N. and M.G.D.; Funding acquisition, P.C., S.Z. and M.G.D.; Investigation, S.M, G. Se., G.S, N.T.L.; Methodology, G.Se, G.S., S.M. Project administration, P.C., S.Z., H.T.N. and M.G.D.; Resources, P.C., S.Z. and M.G.D.; Software, G.S., S.M., G.Se.; Supervision, G.S., P.C., S.Z. and M.G.D.; Validation, P.C., S.Z.; Visualization, D.U., G.S., A.C., P.C., S.Z. and M.G.D.; Writing – original draft, G.S., S.M., G.Se. and M.G.D.; Writing – review & editing, G.S.

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