

Research Paper

In vitro inhibition of the bovine viral diarrhoea virus by the essential oil of *Ocimum basilicum* (basil) and monoterpenes

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Abstract

The bovine viral diarrhoea virus (BVDV) is suggested as a model for antiviral studies of the hepatitis C virus (HCV). The antiviral activity of the essential oil of *Ocimum basilicum* and the monoterpenes camphor, thymol and 1,8-cineole against BVDV was investigated. The cytotoxicities of the compounds were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test, and the antiviral activities were tested by the plaque reduction assay. The oil or compounds were added to the assay in three different time points: a) pre-treatment of the virus (virucidal assay); b) pre-treatment of the cells; or c) post-treatment of the cells (after virus inoculation). The percentage of plaques inhibition for each compound was determined based on the number of plaques in the viral control. The results were expressed by CC₅₀ (50% cytotoxic concentration), IC₅₀ (inhibitory concentration for 50% of plaques) and SI (selectivity index = CC₅₀/IC₅₀). Camphor (CC₅₀ = 4420.12 µg mL⁻¹) and 1,8-cineole (CC₅₀ = 2996.10 µg mL⁻¹) showed the lowest cytotoxicities and the best antiviral activities (camphor SI = 13.88 and 1,8-cineol SI = 9.05) in the virucidal assay. The higher activities achieved by the monoterpenes in the virucidal assay suggest that these compounds act directly on the viral particle.

Key words: antiviral, camphor, 1,8-cineol, thymol.

Introduction

The bovine viral diarrhoea virus (BVDV), the prototype of the genus *Pestivirus* in the *Flaviviridae* family, is responsible for several clinical manifestations in bovines, including respiratory, gastroenteric and reproductive diseases (Thiel *et al.*, 1996). The BVDV is regarded as a model for the hepatitis C virus (HCV) due to similarities in the virion structures, genome organisations and replication cycles (Buckwold *et al.*, 2003). This knowledge has been applied to antiviral studies *in vitro* because the HCV does not replicate efficiently in cell cultures (Bhattacharyya *et al.*, 2003; Buckwold *et al.*, 2003; Yanagida *et al.*, 2004; Romero *et al.*, 2006; Zhang *et al.*, 2010).

The HCV, the only member of the *Hepacivirus* genus, is the most common cause of chronic hepatitis

throughout the world and the main risk factor for cirrhosis and hepatocellular carcinoma (Liang *et al.*, 2000). It is estimated worldwide that approximately 150 million people live with a chronic HCV infection and that more than 350,000 patients die each year from liver disease associated with the infection (WHO, 2012). A vaccine is not available for the HCV infection, and the treatment is primarily based on a combination drug therapy with interferon- α (IFN- α) and ribavirin (Bhattacharyya *et al.*, 2003). However, this type of therapy is expensive and induces several side effects due to high drug toxicity (Bhattacharyya *et al.*, 2003; Yanagida *et al.*, 2004). Thus, new therapeutic options for the treatment of HCV infections are needed.

Natural products obtained from plants have provided the pharmaceutical industry with a great resource for new therapeutic drugs. Approximately 40% of modern medi-

cines originate from compounds present in natural products that are either extracted from nature or synthesised (Jassim and Naji, 2003). A wide variety of active phytochemicals, including terpenes, terpenoids, aliphatics and aromatics, have been identified as responsible for the biological activities of the natural products (Bakkali *et al.*, 2008). Volatile essential oils, commonly used as food condiments, have exhibited high levels of antiviral activity (Astani *et al.*, 2010; Pilau *et al.*, 2011); however, only a few of these compounds have been examined (Jassim and Naji, 2003).

Essential oils constitute a diverse set of compounds that vary in concentrations (Astani *et al.*, 2010). Monoterpenes are the major components of essential oils, constituting 90% of these oils (Bakkali *et al.*, 2008). The monoterpenes 1,8-cineole, camphor and thymol are found in different concentrations among plants (*e.g.*, *Ocimum basilicum* (basil), *Rosmarinus officinalis* (rosemary) and *Thymus vulgaris* (thyme)) commonly used in gastronomy and traditional medicine (Lee *et al.*, 2005; Pozzatti *et al.*, 2008). The monoterpenes 1,8-cineole and thymol have previously been shown to display antiviral activities against the herpes simplex virus (HSV) *in vitro* (Astani *et al.*, 2010).

Ocimum basilicum L., commonly known as basil, is a plant of the family *Lamiaceae*, which is largely utilised as a cooking herb and which has been applied in traditional medicine for the treatment of assorted diseases, including gastric and respiratory disorders (Sartoratto *et al.*, 2004; Chiang *et al.*, 2005). The potential activities of basil against bacteria (Suppakul *et al.*, 2003; Sartoratto *et al.*, 2004), fungi (Pozzatti *et al.*, 2008) and viruses (Chiang *et al.*, 2005) have been investigated. The chemical composition of the basil plant essential oil includes thymol, methyl-chavicol, linalool, eugenol, 1,8-cineole and pirenene (Suppakul *et al.*, 2003; Sartoratto *et al.*, 2004; Chiang *et al.*, 2005; Pozzatti *et al.*, 2008) and the composition may vary in quality and quantity.

The present study was conducted to test the antiviral activities of the essential oil of *Ocimum basilicum* (basil) and its constituent compounds 1,8-cineole, camphor and thymol against BVDV in different time points of the infection.

Materials and Methods

Essential oils and monoterpenes

The procedures for obtaining the plant samples (*Ocimum basilicum*), essential oil extraction and analyses of the chemical constituents were previously reported (Pozzatti *et al.*, 2008). The qualitative and semiquantitative analyses of the chemical constituents of basil were performed using the techniques of gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS), which yielded the mass spectra of most essential oil components (Pozzatti *et al.*, 2008). The compounds

1,8-cineole, camphor and thymol were purchased commercially (Acros Organics, Belgium).

To perform the activity tests, the essential oil of basil and the monoterpenes were first diluted in methanol to a concentration of 0.64 g mL⁻¹ (solution I). Solution I was then diluted to 1:100 in minimal essential medium (MEM) to a final concentration of 6400 µg mL⁻¹ (solution II). Solution II was used as the working dilution in the cytotoxicity and antiviral activity tests.

Cell culture and viruses

Madin-Darby bovine kidney cells (MDBK) were maintained in MEM with 10% equine serum (ES) and penicillin, streptomycin and amphotericin B at the concentrations of 100 U mL⁻¹, 100 µg mL⁻¹ and 2.5 µg mL⁻¹, respectively. Cell cultures were incubated at 37 °C with an atmosphere of 5% CO₂. The cytotoxicity and antiviral activity tests were performed in 96 well- and 6 well-plates, respectively.

The bovine viral diarrhoea virus (BVDV) cytopathic strain Singer, genotype 1 was provided by the Setor de Virologia of the Universidade Federal de Santa Maria (UFSM). The virus stocks were prepared and titrated as previously described (Botton *et al.*, 1998), and aliquots were kept at -70 °C until use.

Cytotoxicity assay

The cytotoxicities of the essential oil of *O. basilicum* and the monoterpenes were measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay (M2128, Sigma-Aldrich, Inc, St. Louis, MO, USA), according to the methodology described by Mosmann (1983) and modified by Cueto *et al.* (2011).

Briefly, MEM and increasing concentrations (25 to 3200 µg mL⁻¹) of the essential oil or compounds were added to the preformed cell monolayer (1.6 x 10⁵ cells mL⁻¹) in 96-well plates (NUNC, Roskilde, Denmark) for a total of 6 replicates for each concentration. Control wells for cells only and methanol toxicity were included. After 72 h of incubation at 37 °C and 5% CO₂, the medium was removed, 50 µL of MTT (1 mg mL⁻¹) was added to the plates and the plates incubated at the same conditions for an additional period of 2 h. After the removal of the MTT, 100 µL of dimethyl sulfoxide (DMSO) was added to solubilise the formazan crystals. The supernatant was then transferred to a new plate, and the absorbancies were read on a spectrophotometer at 540 nm.

The cell viability was calculated according to the formula: absorbance of the compound or essential oil/absorbance of the cell control x 100%. The concentration-effect curve was obtained by a linear regression analysis using data from three independent experiments performed in duplicate. The concentration of the compound that reduced cell viability by 50% was defined as the 50% cytotoxic concentration (CC₅₀). The highest concentration of the com-

pound that did not cause cytotoxicity (maximum non-toxic concentration = MNTC) was subsequently used in the tests for antiviral activity.

Antiviral assay

The antiviral activities for each compound were measured by a plaque reduction assay. Three different assays were performed to elucidate the mechanisms of action for the oil and the monoterpenes. Treatment with the essential oil or monoterpenes was applied at different time points: a) pre-treatment of the virus (virucidal assay); b) pre-treatment of the cells; or c) post-treatment of the cells, as described by Astani *et al.* (2010) with some modifications.

The virucidal assay was performed with a constant volume of a virus suspension (BVDV) and varying concentrations of the essential oil or monoterpenes, and the mixtures were incubated in microcentrifuge tubes for 1 h at room temperature. Following the incubation period, aliquots of each mixture were inoculated into the cells and viral adsorption was allowed to proceed for 2 h at 37 °C (10^3 PFU). The residual inoculum was removed, and the infected cells were then overlaid with 2 mL of MEM containing 0.5% agarose and 5% equine serum. The plates were incubated for 72 h at 37 °C, followed by fixation and staining with formaldehyde and crystal violet for 1 h at room temperature. Finally, the crystal violet was removed and plaque counting was performed. The percentage of virus inhibition for each concentration of a compound was calculated based on the plaque counts observed in treated cells compared to the virus control monolayers (untreated cultures). Thus, the concentration of the essential oil or monoterpenes that inhibited the plaque count by 50% (inhibitory concentration for 50% of plaques = IC_{50}) was determined from the dose-response curves. The selectivity index (SI) was calculated according to the ratio CC_{50}/IC_{50} .

The procedures to perform the pre- and post-treatment assays were similar to that described for the virucidal assay. For the pre-treatment assay, the cells were incubated with the MNTC of the essential oil or monoterpenes for 1 h prior to virus inoculation. After this incubation period, the compound was removed and the virus inoculation was performed as described above. For the post-treatment assay, the essential oil or monoterpenes were added after the virus inoculation and incubated with the cells for a period of 72 h at 37 °C and 5% CO_2 . Control wells containing MEM with 1% methanol without the oil or monoterpenes were also included. All results were calculated as the average of three independent experiments performed in duplicate.

Results and Discussion

The chromatographic analysis of the basil essential oil used to perform the present study was previously described (Pozzatti *et al.*, 2008). The data from the cytoto-

xicity and antiviral tests are described in Table 1 and Figures 1, 2 and 3.

The main constituents of the essential oil of *Ocimum basilicum* used in this study were identified as monoterpenes: 1,8-cineole (23.61%), camphor (12.80%), and linalool (31.22%) (Pozzatti *et al.*, 2008). The amount of the

Table 1 - Cytotoxicity and antiviral activity of the essential oil of *Ocimum basilicum* and monoterpenes against BVDV. The results were obtained from the average of three independent experiments performed in duplicate.

Essential oil/monoterpene	$CC_{50}^a \pm SD^b$	$IC_{50}^c \pm SD$	SI ^d
Essential oil of <i>Ocimum basilicum</i>	1750.01 \pm 11.32	474.29 \pm 8.65	3.69
1, 8-cineole	2996.10 \pm 9.53	331.17 \pm 7.42	9.05
Camphor	4420.12 \pm 8.89	318.51 \pm 8.57	13.88
Thymol	1404.32 \pm 6.91	248.56 \pm 5.32	5.65

^aCytotoxic concentration ($\mu\text{g/mL}$) for 50% of the cell culture.

^bStandard deviation.

^cInhibitory concentration ($\mu\text{g/mL}$) for 50%, as obtained in the virucidal assay.

^dSelectivity index (CC_{50}/IC_{50}).

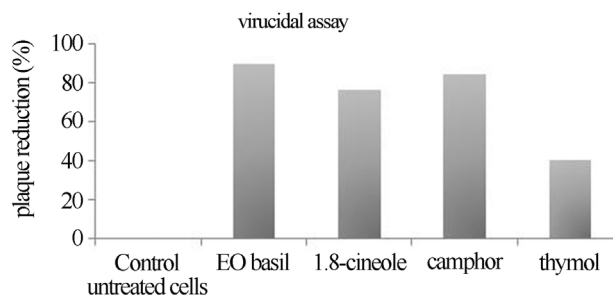


Figure 1 - Antiviral activity of the essential oil (EO) of *Ocimum basilicum* (basil) and monoterpenes against the bovine viral diarrhoea virus (BVDV) in the virucidal assay. The virus was incubated with the MNTCs of the essential oil/ monoterpenes for 1 h at 25 °C. Readings were performed 3 days after the inoculation by comparison with non-inoculated cells. The results are expressed as the percentages of plaque reduction.

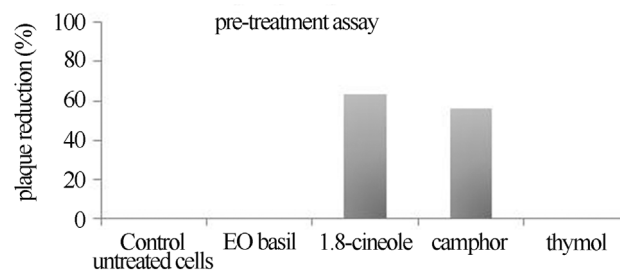


Figure 2 - Antiviral activity of the essential oil (EO) of *Ocimum basilicum* (basil) and monoterpenes against the bovine viral diarrhoea virus (BVDV) after the pre-treatment of cells. The cells were incubated with the MNTCs of the essential oil/ monoterpenes prior to viral infection. Readings were performed 3 days after the inoculation by comparison with non-inoculated cells. The results are expressed as the percentages of plaque reduction.

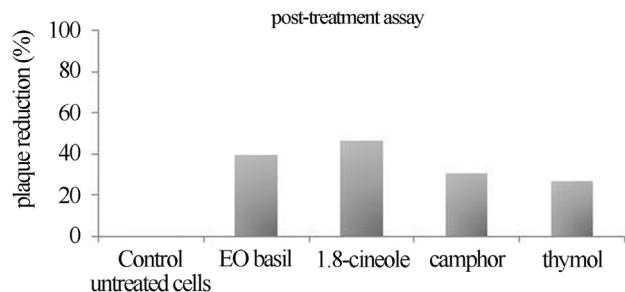


Figure 3 - Antiviral activity of the essential oil (EO) of *Ocimum basilicum* (basil) and monoterpenes against the bovine viral diarrhoea virus (BVDV) after virus inoculation. The MNTCs of the essential oil/ monoterpenes were added to the cells after virus absorption and maintained for 72 h. The readings were then performed by comparison with non-inoculated cells. The results are expressed as the percentages of plaque reduction.

essential oil or compound required to demonstrate a 50% cytotoxic concentration (CC_{50}) was similar, or, in some cases, superior to values described in previous studies using essential oils and constituent compounds. Camphor ($CC_{50} = 4420.12 \mu\text{g mL}^{-1}$) and 1,8-cineole ($CC_{50} = 2996.10 \mu\text{g mL}^{-1}$) demonstrated lower toxicities towards the MDBK cells than the essential oil of basil ($CC_{50} = 1750.01 \mu\text{g mL}^{-1}$), while thymol ($CC_{50} = 1404.32 \mu\text{g mL}^{-1}$) was the most toxic of all the products examined (Table 1). The values of CC_{50} demonstrated for the essential oil of *Lippia graveolens* (Mexican oregano) and its major constituent, carvacrol, toward the same cell culture have been reported as 568 and $215 \mu\text{g mL}^{-1}$, respectively (Pilau *et al.*, 2011). Moreover, propolis extracts from two different sources have been shown to cause high toxicity to MDBK cells, exhibiting CC_{50} values of 293 and $343 \mu\text{g mL}^{-1}$ (Cueto *et al.*, 2011).

1,8-cineole has previously been tested for antiviral activity against the herpes simplex virus, exhibiting a CC_{50} value of $2000 \mu\text{g mL}^{-1}$ (Astani *et al.*, 2010). In the same study, the CC_{50} for thymol was $85 \mu\text{g mL}^{-1}$. It is important to emphasise that the cells utilised to perform these experiments were RC-37 in the referenced study (Astani *et al.*, 2010). Different experimental conditions and the use of diverse cell cultures to perform such experiments may be the reason for the differences observed. However, there are relevant aspects of the studies that should be considered. The cytotoxicities of the monoterpenes, 1,8-cineole and thymol, demonstrated consistency when tested against different cell lines in independent laboratories. Low toxicity to the cell culture is a good initial indicator for an antiviral candidate; thus, the data obtained are promising because the oil and compounds examined in this study demonstrated lower toxicities in almost all of the comparisons.

The parameters for the definition of a compound as a good antiviral candidate are not well defined, showing a great variation among values of IC_{50} and SI for different viruses and products. There are numerous variables that in-

fluence the final results of a susceptibility test, including cell culture, virus titer, incubation times, concentration of the antiviral compound, reference strains, assay methods, calculations and interpretation criteria (Swierkosz and Hodinka, 1999). However, the basis of our discussion will be a suggestion from Amoros *et al.* (1992) applied to herpes simplex viruses (HSV), which propose that a selectivity index (SI) greater than four would be appropriate to consider that a compound has a potential antiviral activity.

Following the guidelines cited above, it could generally be concluded that the monoterpenes examined showed antiviral activities towards BVDV (Table 1). In particular, the monoterpenes, camphor (SI = 13.88) and 1,8-cineole (SI = 9.05) presented the highest SI values in the virucidal assay. On the other hand, the IC_{50} and SI of the *Ocimum basilicum* essential oil were not acceptable, despite the considerable percentage of viral inhibition shown by this essential oil (Table 1).

The antimicrobial activity of the essential oil of *O. basilicum* has partly been attributed to the presence of a high concentration of linalool in tested samples (Suppakul *et al.*, 2003; Sartoratto *et al.*, 2004). The concentration of this monoterpene in the essential oil samples for this study was 31.22%, which may be an insufficient amount to demonstrate expressive antiviral activity. Additionally, camphor was not present in a major concentration in this sample of the essential oil. When analysed separately, camphor and 1,8-cineole exhibited high values of SI, which may suggest the presence of a constituent compound in a minor concentration acting synergistically with these monoterpenes for an antagonistic effect when the essential oil of *O. basilicum* as a whole was examined.

Although the results indicate a high percentage of plaque reduction (90%) by the essential oil of *O. basilicum*, the doses needed to inhibit plaque formation ($IC_{50} = 474.29 \mu\text{g mL}^{-1}$) (Table 1) make it impracticable to use this essential oil as an antiviral therapy. In addition, extracts from this plant did not demonstrate antiviral activity against HCV when examined by protease inhibition experiments in previous work (Hussein *et al.*, 2000). Extracts from the leaves of *O. basilicum* also exhibited low percentages of virus inhibition (59.8% for the alcoholic extract and 37.6% for the aqueous extract) in a study that tested different plants commonly used in traditional Sudanese medicine (Hussein *et al.*, 2000). Thus, the evidence indicates that the essential oil and extracts of *O. basilicum* would not have an antiviral effect on HCV; though, further studies are needed. Conversely, the results obtained from the essential oil compounds studied individually indicate antiviral activities.

The infectivity of the BVDV was greatly reduced by the treatment of the virus with the monoterpenes for 1 h prior to inoculation (Figure 1). At maximum non-toxic concentrations (MNTC), the observed plaque reductions were approximately 84% for the camphor and 75% for 1,8-cineole. The plaque formation by HSV-1 was also reduced

when the virus was incubated for 1 h at 37 °C with 1,8-cineole (Astani *et al.*, 2010), although the percentage of reduction (40%) was considered moderate. In another experiment, 1,8-cineole and camphor reduced the infectivity of HSV-1 by 35% and 0%, respectively, when used at the dilution of 0.1% (Sivropoulou *et al.*, 1997). The viruses from the *Herpesviridae* family are viruses showing an expressive and diverse number of proteins at the envelope (Roizmann *et al.*, 1992), while the *Flaviviridae* family viruses are relatively smaller showing less diversity in envelope proteins (Ridpath, 2010) than the herpes viruses. These structural differences most likely contributed to the diversity of results observed in this study.

The pre-treatment of cells with the compounds (Figure 2) or the incubation of these compounds with the cells after virus inoculation (Figure 3) did not result in antiviral activities at the same level as the direct treatment of the virus (Figure 1). These results suggest that the investigated compounds acted directly on the viral particle and interfered with viral infectivity. The antimicrobial actions of the essential oils and its main constituents have previously been explained based on their effects on the structure and functions of the bacterial membrane. The activities of these compounds were attributed to their lipophilic characteristics that would allow their interaction with the lipidic membrane of the bacteria (Helander *et al.*, 1998; Cox *et al.*, 2000). A similar effect could be expected with the lipidic viral envelope (Schnitzler *et al.*, 2007). In fact, several essential oils and their major compounds have demonstrated antiviral activities against viruses with a lipidic envelope, suggesting that these compounds inactivated the virus by directly interfering with the viral envelope structures or masking viral structures needed for the absorption and entry to the cell (Schnitzler *et al.*, 2007; Astani *et al.*, 2010).

In general, thymol was the least efficient monoterpene in the antiviral activity tests performed (Figures 1, 2, and 3). In another virucidal assay previously described, this monoterpene inhibited the plaque formation of HSV by > 80% (Astani *et al.*, 2010). However, the therapeutic index of the compound in that case was only 2.8 due to its high toxicity to the RC-37 cells used to perform the tests.

Studies have demonstrated the potential of medicinal plants for therapeutic applications using the BVDV as a model for the HCV. The analysis of the antiviral effect of artemisina, obtained from the plant *Artemisia annua*, has suggested a potential use for this compound in association with current therapies for the infections caused by flaviviruses (Romero *et al.*, 2006). Aqueous extracts from *Celosia cristata*, *Ophioglossum vulgatum*, *Houttuynia cordata*, *Selaginella tamariscina*, *Alpinia galanga* and *Alpinia oxyphylla* also inhibited BVDV, while not showing toxic effects on the MDBK cells (Herrmann *et al.*, 2011). In addition, extracts from the roots of *Phyllanthus amarus* caused a significant reduction in the cytopathic effect of BVDV with a therapeutic index greater than 6 and no

cytotoxicity, which has justified a more detailed investigation of a possible action on the HCV (Bhattacharyya *et al.*, 2003).

The comparisons among the indexes already described and obtained from the monoterpenes utilised in the present study indicate potential anti-BVDV activities for camphor and 1,8-cineole (Table 1). The antiviral activities against the BVDV exhibited by the monoterpenes in this study, especially camphor, may suggest that these compounds could have activity on the HCV, since the BVDV virus has been utilised as a model for studies on HCV (Bhattacharyya *et al.*, 2003; Buckwold *et al.*, 2003; Yanagida *et al.*, 2004; Zhang *et al.*, 2010). However, more studies are needed in order to demonstrate such possibility.

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