Inhibitory effect of Ocotea quixos (Lam.) Kosterm. and Piper aduncum L. essential oils from Ecuador on West Nile virus infection.
Inhibitory effect of *Ocotea quixos* (Lam.) Kosterm. and *Piper aduncum* L. essential oils from Ecuador on West Nile virus infection.

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Abstract

West Nile virus (WNV) is a mosquito-borne flavivirus responsible of neuroinvasive manifestations. Natural products are well-known for their biological activities and pharmaceutical application. In this study the inhibitory effects of essential oils (EOs) of Ocotea quixos (Lam.) Kosterm. and Piper aduncum L. on WNV replication were investigated.

WNV was incubated with EOs before adsorption on Vero cells, viral replication was carried out in the absence or presence of EO. Cells were exposed to EO before the adsorption of untreated-virus. GC-MS and GC-FID were used for chemical characterization of EOs.

Cell protection from infection was observed for both EOs. P. aduncum EO was characterized by dillapiole as main compound (48.21%) and O. quixos EO by 1,8-cineole (39.15%).

O. quixos and P. aduncum should be considered for further investigations, such as the study of molecular and cellular mechanisms of action and in vivo evaluation for the development of a compound against WNV.

Further investigations, such as the study of molecular and cellular mechanisms of action and in vivo evaluation, should be performed on these essential oils to derive new potential drugs against WNV.
Keywords: Ocotea quixos, Piper aduncum, essential oil, West Nile virus, antiviral activity, Ecuadorian Amazon Region.

Introduction

West Nile virus (WNV) is a mosquito-borne flavivirus, family Flaviviridae, widely distributed throughout Africa, the Middle East, Asia, Southern Europe, Australia and the Americas. The virus was originally isolated from Uganda in 1937. WNV caused epidemic outbreaks in Asia, Europe and Australia and, in the 1999, it was introduced into the United States where it became endemic (Saxena et al. 2017). While birds serve as amplifier hosts, mosquitoes of the Culex genus function as vectors. Humans and horses are dead-end hosts (David and Abraham 2016). WNV causes asymptomatic infections in 80% of cases, while 19% of patients develop flu-like illnesses. In less than 1% of the symptomatic individuals, virus entry into the central nervous system (CNS) results in neuroinvasive manifestations, such as meningitis, encephalitis, poliomyelitis, and death (Gubler 2007). Despite the important impact of WNV infection-associated diseases on human health, there are currently no available human vaccines or specific antiviral therapies for this disease. To date, only a West Nile vaccine is available for horses. Natural products are widely used all over the world as treatment for many diseases and deep investigations are justified from several studies (Newman and Cragg 2007; Bhalla et al. 2013; Atanasov et al. 2015). A recent study demonstrated the antiviral potential of different polyphenols present in plants and natural products, such as wine and tea, against WNV, by affecting the attachment and entry steps of the virus life-cycle (Vázquez-Calvo et al. 2017). Further studies on the antiviral potential of
natural compounds against WNV are not reported to date. Moreover, Ecuador belongs
to a selected group of 17 countries defined “Megadiverse” due to its impressive
biological diversity (Mittermeier et al. 1999; Sierra et al. 2002), which is an important
source of bioactive compounds. EOs from Ecuadorian Amazon region (EAR) have been
investigated in the last decades in order to deepen their biological activities (Bruni et al.
2004; Tognolini et al. 2006; Sacchetti et al. 2006; Scalvenzi et al. 2007; Guerrini et al.
2014).

To our knowledge, studies evaluating antiviral effect of *O. quixos* (Lam.) Kosterm.
(Lauraceae) and *P. aduncum* L. (Piperaceae) EOs against WNV have not been reported.

*O. quixos* (Lam.) Kosterm belongs to the Lauraceae family. It is traditionally used as
ingredient for infusions and beverages or as a flavoring for foods (Naranjo 1981;
Friedman et al. 1993). Although few studies have been carried out on the biological
properties of *O. quixos*, *in vivo* and *in vitro* investigations on this plant highlighted
significant anti-inflammatory activity of its EO (Ballabeni et al. 2009).

*P. aduncum*, from Piperaceae family, is well-known in folk medicine for the
antimicrobial and insecticide activities and for the treatment of dysentery and wound
healing (Durant-Archibold et al. 2018).

Many studies have been performed on *P. aduncum* demonstrating efficacy of as
insect repellent (Mamood et al. 2017) and methanolic extract as antiviral plant-derived
product (Lohézic-Le Dévèhat et al. 2002). Moreover the importance of the *P. aduncum*
EO has been demonstrated also against *P. falciparum* indicating that this EO could be a
promising antimalarial agent (Monzote et al. 2017). In an effort to identify antiviral
therapies effective against WNV, in this study potential antiviral effect of *O. quixos* and *P.
aduncum* EOs were tested *in vitro*.

**Methods**
Material and Methods

**Plant material**

Fresh leaves of *O. quixos* (*Canela amazónica*) and *P. aduncum* (*Matiko*) plants were collected from a wild population in the Amazonian region of Pastaza (Ecuador) in June 2016. Species authentication were certified by Dr. David Neill and voucher specimens from each plant were deposited at the Herbarium ECUAMZ of the Amazonian State University (UEA) in Ecuador (voucher specimen: Neill 18070B, Scalvenzi 18070C).

**Isolation of EOs**

The EOs were obtained by hydrodistillation in a stainless steel distiller equipped with a Clevenger apparatus, performing three distinct distillations for 4 h. All samples for each EO were gathered, dried over anhydrous sodium sulphate and stored in sealed amber vials at 4°C.

**Gas-chromatographic analysis of EOs**

The compound identification was realized by GC-MS analysis and the quantification of individual components was performed by GC-FID, calculating the relative peak average area of three separated injections. The instrument used for quantitative determination was a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector and a Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethylsiloxane column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm). Operating conditions were as follows: injector temperature 300°C, FID temperature 300°C, carrier (Helium) flow rate 1 mL/min and split ratio 1:50. The initial oven temperature was 55°C and then raised to
100°C at a rate of 1°C/min, then raised to 250°C at a rate of 5°C/min and then kept constant at 250°C for 15 min. One microliter for each replicate was dissolved in CH$_2$Cl$_2$ (Sigma-Aldrich) and injected. The EOs percentage composition was computed by the normalization method from the GC peak areas, without using correction factors. The compound identification of EOs were performed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library. The conditions were the same described for GC analysis and also the same column was used. The mass spectrometry conditions were as follows: ionization voltage, 70 eV; emission current, 10 µAmp; scan rate, 1 scan/s; mass range, 29-400 Da; trap temperature, 150°C, transfer line temperature, 300°C. The EO compounds were characterized by comparing their linear retention indices and the MS fragmentation pattern with those of other known EOs, with pure compounds and by matching the MS fragmentations patterns and linear retention indices with the above mentioned mass spectra libraries and with those in the literature (Adams 2007). The linear retention index of each component was determined adding a C$_8$-C$_{32}$ n-alkanes (Sigma-Aldrich) to the EO before injecting in the GC-MS equipment and analyzed under the same conditions reported above (Guerrini et al. 2014)[14].

Preparation of mother solution for biological assays

An aliquot of dimethylsulfoxide (DMSO; Sigma–Aldrich) was added to the stock solution of each EO in order to obtain a 84mg/mL concentration range. Serial dilutions of the DMSO/EO solution were made with virus dilution buffer for the infection and with MEM maintenance medium for maintaining.
Cell culture and virus

African green monkey kidney (Vero) cells were grown at 37° C in a humidified atmosphere, with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Lonza, Milan, Italy) supplemented with 10% inactivated fetal calf serum (FCS, Flow Laboratories, Irvine, UK.), 2 mM glutamine, 2% non-essential amino acids (Gibco, Paisley, UK), penicillin (100 IU/mL), and streptomycin (100 µg/mL). WNV strain, lineage 1, isolated from a patient during the WNV outbreak occurred in Sardinia Region in 2011 (Magurano et al. 2012) [15] was used for the study. For virus production, monolayers of Vero cells in 75-cm² tissue culture flasks were infected with WNV. After 5 days at 37° C, infected cells were harvested with freeze-and-thaw cycle, and cellular debris was removed with low-speed centrifugation, and virus titer was measured by standard plaque assay. The virus was stored at −80° C until used.

Plaque assay

WNV titer was determined by plaque assays in Vero cells growing in 24-well plates. Briefly, serial tenfold dilutions of the viral suspension were added (0.1 mL/well) in duplicate. After infection, the cells were incubated for 1 h at 37°C. Subsequently, Tragacanth gum powder (SIGMA cat. G1128-100G) supplemented 1:1 with DMEM medium with 5% inactivated FBS, 2 mM glutamine, 2% non-essential amino acids, penicillin (100 IU/mL), and streptomycin (100 µg/mL) was added, and the plate was incubated for 5 days at 37° C. The viral plaques were visualized by 1% crystal violet solution (Fortuna et al. 2015). The titer was estimated by counting the number of
plaques observed in each well and expressed as plaque-formation unit per milliliter (p.f.u./mL) (viral titer: $6.93 \times 10^6$ p.f.u./mL).

**Cytotoxicity assay**

A cytotoxicity test was performed for all EOs. Each EO was diluted 1:10 in DMSO and subsequent several dilutions were made in MEM medium. Vero cell monolayers were put in contact with the mixture of MEM medium plus oil and maintained at $37^\circ$ C with 5% CO2. The cells were monitored daily to check the EO toxicity. After 24 h, the following parameters were evaluated: cell morphology and viability (determined by neutral red staining) were examined by light microscopy and cell proliferation was evaluated quantitatively by microscopic counts after dispersion into individual cells with trypsin. EO dilutions that did not affect any of these parameters were considered as non-cytotoxic concentrations and utilized for antiviral assays. For neutral red staining the 50% cytotoxic concentration (CC$_{50}$) was defined as the concentration that reduces the optical density (OD) of treated cells to 50% with respect to untreated cells (Pietrantoni et al. 2015).

**Dose-Response Assay**

The antiviral activity of *O. quixos* and *P. aduncum* EOs was assayed by neutral red assay. Briefly, in 96-well culture plate, monolayer cultures of Vero cells were incubated with different concentrations of *O. quixos* and *P. aduncum* starting from 2.6 µg/mL during the virus attachment step (1h 4°C). As viral inoculum was utilized WNV at a multiplicity of infection (m.o.i.) of 0.1 p.f.u./cell. After adsorption, Vero cells were
rinsed thoroughly and incubated with the same concentrations of EO. The cells were maintained at 37°C in 5% CO₂. The cytopathic effect (CPE) induced by WNV was measured 72 hours after infection by the neutral red uptake assay. Briefly, treated and untreated cells were stained for 3 h at 37°C with neutral red (0 mg/mL), thereafter cells were washed with Phosphate Buffered Saline (PBS) and fixed with 4% formaldehyde, 10% CaCl₂. The uptaken dye was extracted by 1% acetic acid in 50% ethanol and the optical density was measured at 540 nm in a spectrophotometer (Pietrantoni et al. 2015). Results were expressed as percentage of cell viability with untreated infected control cultures. The concentration that reduced the absorbance of infected cells to 50% when compared to cell and virus controls was considered the effective concentration (EC₅₀). The EC₅₀ was calculated according to the following equation: \[\frac{(A - B)}{(C - B) \times 100}\], where A is the control sample absorbance, B is the cell control absorbance, and C is the virus control absorbance. The selectivity index (SI) was calculated using the CC₅₀ and EC₅₀ data and applying the formula SI = CC₅₀/EC₅₀.

Antiviral activity

The antiviral activity of the EOs was measured using the neutral red assay as already described. The antiviral assays were performed at 24 h after seeding, using confluent Vero cell monolayers cultured in 96-well plates. EOs and components were always used at the non-cytotoxic concentration as follows (33.6, 16.8, 2.1, 1 µg/mL). Cells without the EO were used as a control. The EOs were included in different time points as follows:

i. To evaluate the presence of virucidal activity, direct inactivation of WNV by the extracts was tested. Viral inoculum was mixed with varying concentrations of the EO
incubated at 37°C in 5% CO₂ for 1 h. After that, viral inoculum was used to infect
monolayer cultures of Vero cells, at a m.o.i. of 0.1 p.f.u./cell. After incubation at 37°C
in 5% CO₂ for 1 h, the cells were washed and DMEM maintenance medium was added
and the cells were maintained at 37°C in 5% CO₂.

ii. Cell monolayers were pre-treated 1 h at 37°C with EO prior to inoculation with virus
by adding the EO at varying concentrations. After pre-treatment, the EO was removed
and cells were infected with virus at a m.o.i. of 0.1 p.f.u./cell and incubated at 37°C in
5% CO₂ for 1 h. Then MEM maintenance medium was added and the cells were
maintained at 37°C in 5% CO₂.

iii. Experiments were also performed to determine the viral inhibitory effect of the
selected EOs during the adsorption step. Vero cells grown in 96-well plates were
infected in triplicate with 0.1 p.f.u./cell of virus and EO at concentration described
above.

For all the treatments the neutral Red assay procedure was performed 72 h later,
according to the protocol described in cell viability assay (Pietrantoni et al. 2015).

Statistical analysis

The experiments were performed in triplicate. Relative standard deviations and
statistical significance (Student’s t test; p ≤ 0.05) were calculated using software
STATISTICA 6.0 (StatSoft Italia srl).

Results

Chemical composition of EOs
The yield of distillation for *O. quixos* EO was 0.13±0.01% (w/v). The main component (Table 1) was represented by 1,8-cineole (39.15%), followed in less amount by α-terpineol (7.65%), sabinene (6.46%), α-pinene (6.27%), p-cymene (6.12%), E-caryophyllene (4.73%), terpinen-4-ol (4.22%). Monoterpenes represented the main fraction of EO. Methyl cinnnamate, a characteristic phenylpropanoid of floral calice EO (Bruni et al 2004), was a minor compound in EO obtained from leaves (1.53%). For *P. aduncum* EO, the yield was 0.16±0.01% (w/v). The phenylpropanoid dillapiole (48.21%), trans cedrene (7.53%) and E-caryophyllene (4.80%) were the main compounds.

**Cytotoxicity of EOs**

A neutral red assay was used to determine the cytotoxicity effect of *O. quixos* and *P. aduncum* on Vero cells in which the half maximal cytotoxic concentration (CC50) value of each compound was calculated. Results illustrate a cytotoxic value of CC50 = 163 µg/mL for *P. aduncum* compared to *O. quixos* with CC50 = 840 µg/mL. Treated cells with vehicle control, 1% DMSO did not show any cytotoxicity against Vero cells.

**Dose-Response Assay**

In order to evaluate the effect of *O. quixos* and *P. aduncum* on viral replication, Vero cells were infected with WNV at 0.1 m.o.i. p.f.u./cell and exposed to various two-folds concentrations of EO starting from 2.6 µg/ml for 72 h post-infection (p.i.). Results are presented in Fig 1 as percentage of cell viability and represent the average of three independent experiments. As showed in the figure both EOs were able to inhibit...
viral replication but *O. quixos* was more effective at concentration of 268 and 134.4 µg/ml respect *P. aduncum* that showed a viral inhibition similar for all concentration. The 50% effective concentration (EC50) for *O. quixos* was 372 µg/ml with a selectivity index (SI) of 2.2 as showed on (Table 2).

Antiviral activity of *O. quixos* on WNV

In order to better investigate the inhibitory effects of *O. quixos* on WNV, EO was added at different stages during viral infection. As showed on Fig 2 (A), pre-treatment of virus with *O. quixos* showed relevant virucidal activity at concentrations of 33.6 µg/ml and 16.8 µg/ml. The EO was also effective at these concentrations when host cells were pre-treated with drugs prior to infection, showing a percentage of cell vitality of 60% (Fig 2 (B)). Same results were obtained when *O. quixos* EO at concentration of 33.6 µg/ml and 16.8 µg/ml was present during the adsorption step (Fig 2 (C)). We, therefore, observed similar inhibition effect on viral replication in all three conditions studied, when the EO was used at the concentrations of 33.6 µg/ml and 16.8 µg/ml, with a percentage of cell viability around 60%. No relevant inhibition was observed when the WNV was pre-treated with EO at the concentrations of 2.1 µg/ml and 1 µg/ml, for all conditions used (Fig 2 (A,B,C)).

Antiviral activity of *P. aduncum* on WNV

As showed on Fig 3 (A) antiviral activity of the *P. aduncum* was most pronounced when viruses were treated before inoculation showing a percentage of cell viability of 79% at the concentrations of 33.6 µg/ml, 88% at 16.8 µg/ml, 87% and 78% at 2.1 an 1 µg/ml, respectively. When cells were incubated with EO only 1 µg/ml showed a small protection (68% of cell viability) while no relevant antiviral activity was showed by the
other concentrations (Fig 3(B)). When the EO was added during infection, the results showed an antiviral activity at all the concentrations with a percentage of cell viability of around 68% at 1 µg/ml (Fig 3(C)).

Discussion and conclusion

Viral diseases are still a major problem for human health worldwide. Although natural products have inherently high chemical diversity, plant-based products or bioactive pure compounds obtained from EOs may be a new source of antiviral drugs but a few number of studies focus on this research field. So far, only a limited number of drugs are effective against many of these viruses, which has prompted research into finding new antiviral lead molecules (Li et al. 2013, Elizáquível et al. 2013, Tanu and Harper 2016, Schnitzler et al. 2007, Astani et al. 2010, Lohézie Le Dévéhat et al. 2002, Ocazionez et al. 2010, Swamy et al. 2016). *P. aduncum* EO has been investigated by several authors mainly focusing chemical characterization, antimicrobial, insecticidal, larvicidal and anti-protozoic (Guerrini et al. 2009, Bernuci et al. 2016, Oliveira et al. 2013, Villamizar et al. 2017, Ling A et al. 2009, Monzote et al. 2017), but no data are available regarding antiviral activity. Also *O. quixos* EO was characterized and tested for its antimicrobial, antiplatelet and antithrombotic activity (Sacchetti et al. 2006, Naranjo 1981, Rolli et al. 2014, Tognolini et al. 2006, Ballabeni et al. 2007); from our knowledge no antiviral activities test were performed until now. The limited efficacy of the current treatment of WNV infection enhances the need for novel therapies that include substances with innovative viral targets and/or mechanisms of action. Our study has been performed to analyse the potential capacity of *O. quixos* and *P. aduncum* EOs, collected in the Ecuadorian Amazon, to reduce the WNV replication in infected cells.
The chemical composition of leaf *O. quixos* EO, showing 1,8-cineole (39.15%) as main component, cinnamate derivatives and E-caryophyllene among minor compounds, did not reflect our previous results (Sacchetti et al. 2006), where E-caryophyllene, cinnamyl acetate and other derivatives were the characteristic molecules. The yield was instead comparable with our previous results. However, it should be noted that the variation of chemical composition for this EO has not yet been studied extensively. *P. aduncum* EO showed an overlapping composition to our previously data (Guerrini et al. 2009), with small quantitative differences regarding minor compounds: furthermore, germacrene D (3.05%) was not detected in the previous studies. *P. aduncum* EO have been largely studied in the last two decades and the formation of two chemotypes by different biosynthetic routes has been evidenced. In fact, according to our data, Maia et al. (1998), Cieció and Ballestero (1997), Fazolin et al. (2007), De Almeida et al. (2009) isolated EOs from leaves of *P. aduncum* in different localities of Amazonian region and determined that dillapiole, formed by the shikimate pathway, was the main compound with a variability from 31.5% to 97.3%. The study of *P. aduncum* cultivation in Western Amazonian region confirmed dillapiole as major component (Silva et al. 2014). If the leaves were instead collected from species in Atlantic Forest, and Northeastern and Southeastern Brazil, terpene compounds such as (E)nerolidol and linalool were detected as main components (De Almeida et al. 2009, De Oliveira et al. 2006, Navickiene et al. 2006). The yield of dillapiole-chemotype EO in literature ranged from 0.35% to 4.0% (Guerrini et al. 2009, Fazolin et al. 2007, De Almeida et al. 2009, Silva et al. 2014, Rali et al. 2007), our results instead showed a lower level (0.16%).

EOs were tested for their cytotoxicity on Vero cells, prior to the determination of their inhibitory effect against WNV. *P. aduncum* resulted relatively more toxic than *O.*
The potential antiviral effect of different EOs was determined against WNV on Vero cells in vitro and both EOs tested exhibited a reduction of infectivity at non-cytotoxic concentration. High antiviral activity was observed for selected EOs when WNV was incubated with this substances prior host cell infection (virucidal activities). WNV is an enveloped virus and the results of our study highlighted that both O. quixos and P. aduncum show virucidal activity. Experiments aimed to assess the antiviral activity of EOs have been most frequently conducted on viruses of the herpes group, enveloped viruses (Novak 2011). Schnitzler et al. (2007) demonstrated a virucidal effect of peppermint oil, when herpes simplex virus was mixed with the essential oil prior to inoculation. The application of tea tree oil, the EO of Melaleuca alternifolia, for the treatment of recurrent herpes labialis has been recently reported (Carson et al. 2001, Schuhmacher et al. 2003). Accordingly, a virus lacking of envelope, like adenovirus, was not affected by eucalyptus EO (Cemelli et al. 2008). Therefore our results suggest that O. quixos and P. aduncum could directly inactivate WNV and might interfere with virion envelope structures or mask viral structures which are necessary for adsorption or entry into host cells. In this study, experiments were also performed to determine the viral inhibitory effect treating the cells with EOs before adsorption. This different approach was important to better investigate on mechanisms of antiviral action of the EOs. In our study, O. quixos showed a protection of the cells from viral infection unlike P. aduncum. These findings would suggest an ability of O. quixos to inhibit viral replication by interfering with the virus binding to the cells. P. aduncum didn’t result protective when added to the cells before inoculum. In this case we might, in some ways, assume that the oil does not act
by competing with the virus for binding to the cell, and that part of its activity relies on
direct inactivation of the viral particles after virus adsorption.

For *O. quixos* we observed a good and similar protection from WNV infection adding
the EO to the DMEM medium during the intracellular replication period, up to an EO
concentration of 16.8 µg/ml. These results suggest that *O. quixos* could act directly on
WNV virus and might interfere with virion envelope structures or mask viral structures,
which are necessary for adsorption or entry into host cells. Thus different mechanisms
of antiviral activity of different EOs and compounds seem to be present.

All together, these results support the potential use of EOs *in toto* from medicinal plants
as agents for the treatment of viral infections. The effectiveness of the EOs from
Amazonia against the viruses tested was variable, but their virucidal properties against
these viruses suggest the application of this type of natural products as disinfectants or
topical medicaments.

Previous studies with EOs from eucalyptus, tea tree and thyme (Astani et al. 2010),
have shown the direct inactivating action of EOs and their components on virion
infectivity (HSV-1), with the exception of 1,8-cineole. In particular, α-pinene, α-
terpineol, terpinen-4-ol and p-cymene, detected in considerable amount in *O. quixos*
EO, revealed a high antiviral activity and could be responsible for inactivation action.

However, in our research, further investigation is required to better elucidate the active
components and their mixture responsible of the inhibitory effect on virions.

Results and Discussion

The limited efficacy of the current treatment of WNV infection enhances the need for
novel therapies that include substances with innovative viral targets and/or mechanisms
of action. So far, only a limited number of plant-derived products are effective against
viruses, which has prompted research into finding new antiviral lead molecules (Lohézic-Le Dévéhat et al. 2002; Schnitzler et al. 2007; Astani et al. 2010; Ocazionez et al. 2010; Elizaquível et al. 2013; Li et al. 2013; Swamy et al. 2016; Tanu and Harper 2016).

Our study has been performed to analyze the potential capacity of *O. quixos* and *P. aduncum* EOs, collected in the Ecuadorian Amazon, to reduce the WNV replication in infected cells. *O. quixos* EO was characterized and tested for its antimicrobial, antiplatelet and antithrombotic activity (Naranjo 1981; Sacchetti et al. 2006; Tognolini et al. 2006; Ballabeni et al. 2007; Rolli et al. 2014); from our knowledge no antiviral activities test were performed until now. *P. aduncum* EO has been investigated by several authors mainly focusing on chemical characterization, antimicrobial, insecticidal, larvicidal and antiprotozoic activities (Guerrini et al. 2009; Ling et al. 2009; Oliveira et al. 2013; Bernuci et al. 2016; Monzote et al. 2017; Villamizar et al. 2017). In addition, *P. aduncum* was found active on Poliovirus (Lohézic-Le Dévéhat et al. 2002).

In the present work the chemical composition of *O. quixos* and *P. aduncum* EOs was determined by GC-MS and GC-FID. The yield of distillation for *O. quixos* EO was 0.13±0.01% (w/v), comparable with our previous results (Sacchetti et al. 2006). The main component was represented by 1,8-cineole (39.15%), followed in less amount by α-terpineol (7.65%), sabinene (6.46%), α-pinene (6.27%), p-cymene (6.12%), E-caryophyllene (4.73%), terpinen-4-ol (4.22%) (Table 1): monoterpenes were therefore the main fraction of EO. This chemical profile did not reflect our previous data (Sacchetti et al. 2006), where E-caryophyllene, cinnamyl acetate and other derivatives were the characteristic molecules. Moreover, methyl cinnamate, a typical phenylpropanoid of floral calyx EO (Bruni et al. 2004), was a minor compound derived
from leaves (1.53%). The chemical characterization of leaf EO has not been studied extensively in literature to date. However, it can be highlighted that high biodiversity of Amazonian region can induce plant secondary metabolism to biosynthetic pathways characterized by diversified molecules that can justify the different chemical profile of EOs derived by the same species (Scalvenzi et al., 2017).

Regarding *P. aduncum* EO, the data were similar to those previously published (Guerrini et al. 2009) with dillapiole (48.21%), *trans*-ocimene (7.53%) and *E*-caryophyllene (4.80%) as main compounds and small quantitative differences on minor compounds and germacrene D (3.05%) that was not detected in the past research. The yield was 0.16±0.01% (w/v). *P. aduncum* EO have been largely studied in literature in the last two decades and the formation of two chemotypes by different biosynthetic routes has been evidenced. In fact, according to our data, different studies on EOs from leaves of *P. aduncum* leaf OEs, “derived from different localities of Amazonian region”, showed that dillapiole, formed by the shikimate pathway, was the main compound with a variability from 31.5% to 97.3% (Cicció and Ballestero 1997, Maia et al. 1998, Fazolin et al. 2007 and De Almeida et al. 2009). In addition, the study of *P. aduncum* cultivation in Western Amazonian region confirmed dillapiole as major component (Silva et al. 2014). If the leaves were instead collected from species in Atlantic Forest, and Northeastern and Southeastern Brazil, terpene compounds such as (E)-nerolidol and linalool were detected as main components (De Oliveira et al. 2006; Debonsi Navickiene et al. 2006; De Almeida et al. 2009). The yield of dillapiole-chemotype EO in literature ranged from 0.35% to 4.0% (Fazolin et al. 2007; Rali et al. 2007; De Almeida et al. 2009; Guerrini et al. 2009; Silva et al. 2014), our results instead showed a lower level (0.16%).
EOs were tested for their cytotoxicity on Vero cells prior to the determination of their inhibitory effect against WNV, by using a neutral red assay in which the half maximal cytotoxic concentration (CC$_{50}$) value of each compound was calculated. *P. aduncum* resulted relatively more toxic than *O. quixos*. Indeed, the results illustrated a CC$_{50}$ value of 163 µg/mL for *P. aduncum* and 840 µg/mL for *O. quixos*. Treated cells with vehicle control, 1% DMSO, did not show any cytotoxicity against Vero cells (Table 2).

In order to evaluate the effect of *O. quixos* and *P. aduncum* on viral replication, Vero cells were infected with WNV at 0.1 m.o.i. p.f.u./cell and exposed to various two-fold concentrations of EO starting from 2.6 µg/mL for 72 h post-infection (p.i.). Results are presented in Figure 1 as percentage of cell viability and represent the average of three independent experiments. As shown in the figure, both EOs were able to inhibit viral replication but *O. quixos* was more effective at concentration of 268 and 134.4 µg/mL respect *P. aduncum* that showed a viral inhibition similar for all concentration. The 50% effective concentration (EC$_{50}$) for *O. quixos* was 372 µg/mL with a selectivity index (SI) of 2.2 (Table 2).

In order to better investigate the inhibitory effects of *O. quixos* and *P. aduncum* on WNV, EOs was added at different stages during viral infection. As shown on Figure 2, no relevant differences between the antiviral activities of OE of *O. quixos* OE were observed in all conditions studied. The highest percentages of cell viability (>60%) were observed when the EO was added to the host cells at the concentrations of 16.8 and 33.6 µg/mL prior to the infection. Similar results were obtained when 33.6 µg/mL of EO were added during the adsorption step. Differently, when the EO was added at low concentrations (1 and 2.1 µg/mL) no relevant antiviral activity was observed in any conditions.
Antiviral activity of the *P. aduncum* was most pronounced when the virus was treated before inoculation (Figure 3A) showing a percentage of cell viability of 79% at the concentrations of 33.6 µg/mL, 88% at 16.8 µg/mL, 87% and 78% at 2.1 and 1 µg/mL, respectively. When cells were incubated with EO only 1 µg/mL showed protection (68% of cell viability) while no relevant antiviral activity was shown by the other concentrations (Figure 3(B)). When the EO was added during infection, the results showed an antiviral activity at all the concentrations with a percentage of cell viability of around 68% at 1 µg/mL (Figure 3(C)).

Results of the present paper showed that the antiviral activity of *P. aduncum* EO had mainly virucidal activity. In literature there aren’t many studies of virucidal effect of EOs on WNV. Experiments aimed to assess the antiviral activity of EOs have been most frequently conducted on viruses of the herpes group, enveloped viruses (Novak 2011). Schnitzler et al. (2007) demonstrated a virucidal effect of peppermint oil, when herpes simplex virus was mixed with the EO prior to inoculation. The application of tea tree oil, the EO of *Melaleuca alternifolia*, for the treatment of recurrent herpes labialis has been recently reported (Carson et al. 2001; Schuhmacher et al. 2003). Concerning antiviral activity on others members of the Flaviviridae family, many studies with dengue virus (DENV) have been reported (Garcia et al. 2003; Duschatzky et al. 2005; Raquel Elvira Ocazionez et al 2010; Klawikkan 2011). These latter data support the hypothesis that *P. aduncum* EO components may directly contribute to the inactivation of viral particles by interfering with envelope or masking viral glycoproteins that are necessary for entering host cells.

Accordingly, a virus lacking of envelope, like adenovirus, was not affected by *Eucalyptus* EO (Cermelli et al. 2008). Therefore our results suggest that *O. quixos* and *P. aduncum* could directly inactivate WNV and might interfere with virion envelope
structures or mask viral structures which are necessary for adsorption or entry into host
cells.

In this study, experiments were also performed to determine the viral inhibitory effect
eating the cells with EOs before adsorption. This different approach was important to
better investigate other mechanisms of antiviral action of the EOs. In our study, *O. quixos*
showed a protection of the cells from viral infection unlike *P. aduncum*.

Previous studies with EOs from eucalyptus, tea tree and thyme have shown the direct
inactivating action of EOs and their components on virion infectivity (HSV-1), with the
exception of 1,8-cineole (Astani et al. 2010). Although 1,8-cineole was the main
component of *O. quixos*, our results showed a good antiviral effect of this EO. In
particular, Astani et al. demonstrated that α-pinene, α-terpineol, terpinen-4-ol and p-
cymene, components detected also in *O. quixos* EO, revealed a high antiviral activity
and could be responsible for inactivation action. These data highlight the need to
analyze the efficacy of the single components of *O. quixos* EO in further studies.

For *O. quixos* we observed a good and similar protection from WNV infection adding
the EO to the DMEM medium during the intracellular replication period, up to an EO
concentration of 16.8 µg/mL. These results suggest that *O. quixos* could act directly on
WNV virus and might interfere with virion envelope structures or mask viral structures,
which are necessary for adsorption or entry into host cells. Thus different mechanisms
of antiviral activity of different EOs and compounds seem to be present.

*P. aduncum* didn’t result protective when added to the cells before inoculum; in this
case we might, in some ways, assume that the oil does not act by competing with the
virus for binding to the cell, and that part of its activity relies on direct inactivation of
the viral particles after virus adsorption.
All together, these results support the potential use of EOs in toto from medicinal plants as agents for the treatment of viral infections. The effectiveness of the EOs from Amazonia against the viruses tested was variable, but their virucidal properties against these viruses suggest the application of this type of natural products as disinfectants or topical medicaments.

However, in our research further investigation is required to better elucidate the active components and their mixture responsible for the inhibitory effect on virions.

Viral diseases are still a major problem for human health worldwide. Although the intrinsic complexity of natural products, the research trends clearly indicate that plant-based products will be among the most important sources of new drugs in the future (Atanasov et al. 2015).

Acknowledgements

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Statistical analysis

The experiments were performed in triplicate and were determined by logarithmic regression curves with 95% confident limits. Relative standard deviations and statistical
significance (Student’s t test; p ≤ 0.05) were calculated using software STATISTICA 6.0 (StatSoft Italia srl)

References


Figure 1. Antiviral activity of *O. quixos* (♦) and *P. aduncum* (■) against WNV during intracellular virus replication. Results are presented as mean of three independent experiments ± SD (p<0.05)
Figure 2. Antiviral activity of *O. quixos* against WNV at 0.1 m.o.i.p.f.u./cell (A) after incubation of virus with different concentrations of EO 1h at 37°C before infection; (B) after pre-treatment of cells with drugs before viral infection 1h at 37°C (C) after treatment with different concentrations of EO during infection. CC = cell control; CV = virus control.
Figure 3. Antiviral activity of *P. aduncum* against WNV at 0.1 m.o.i. p.f.u./cell (A) after incubation of virus with different concentrations of EO 1 h at 37° C before infection; (B) after pre-treatment of cells with drugs before viral infection 1 h at 37° C (C) after treatment with different concentrations of EO during infection. CC = cell control; CV = virus control.
Table 1. Chemical composition of *O. quixos* and *P. aduncum* EOs

<table>
<thead>
<tr>
<th>No.</th>
<th>Component</th>
<th><em>O. quixos</em> (Area %)</th>
<th><em>P. aduncum</em> (Area %)</th>
<th>RI exp</th>
<th>RI lit</th>
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Total identified: 99.42% 98.77%

1 Components are listed in order of elution and their nomenclature is in accordance of the NIST (National Institute of Standards and Technology) library; 2 Relative peak areas ± SEM (standard error media), calculated by GC-FID; 3 RI exp: linear retention indices calculated on a Varian VF-5ms column; 4 RI lit: linear retention indices (Adams et al. 2007).
Table 2. CC$_{50}$ = 50% cytotoxic concentration (µg/mL); EC$_{50}$ = 50% effective concentration (µg/mL); SI$_{50}$ = selectivity index (CC$_{50}$/EC$_{50}$)

<table>
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<tr>
<td>O. quixos</td>
<td>840 µg/mL</td>
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<td>P. aduncum</td>
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