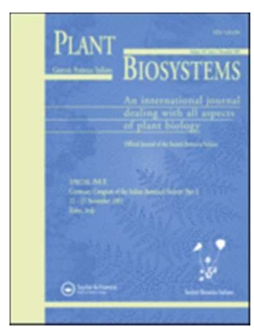

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Inhibitory effect of Ocotea quixos (Lam.) Kosterm. and Piper aduncum L. essential oils from Ecuador on West Nile virus infection.

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Keywords:	<i>Ocotea quixos</i>, <i>Piper aduncum</i>, essential oil, West Nile virus, antiviral activity, Ecuadorian Amazon Region

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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.

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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 deepened 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

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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 effects 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 EO as insect repellent (Mamood et al. 2017) and methanolic extract as antiviral plant-derived product (Lohézic-Le Dévéhat 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 effects of *O. quixos* and *P. aduncum* EOs were tested *in vitro*.

~~**Methods**~~

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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

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137 100°C at a rate of 1°C/min, then raised to 250°C at a rate of 5°C/min and then kept
138 constant at 250°C for 15 min. One microliter for each replicate was dissolved in CH_2Cl_2
139 (Sigma-Aldrich) and injected. The EOs percentage composition was computed by the
140 normalization method from the GC peak areas, without using correction factors. The
141 compound identification of EOs were performed by a Varian GC-3800 gas
142 chromatograph equipped with a Varian MS-4000 mass spectrometer using electron
143 impact and hooked to NIST library. The conditions were the same described for GC
144 analysis and also the same column was used. The mass spectrometry conditions were as
145 follows: ionization voltage, 70 eV; emission current, 10 μAmp; scan rate, 1 scan/s; mass
146 range, 29-400 Da; trap temperature, 150°C, transfer line temperature, 300°C. The EO
147 compounds were characterized by comparing their linear retention indices and the MS
148 fragmentation pattern with those of other known EOs, with pure compounds and by
149 matching the MS fragmentations patterns and linear retention indices with the above
150 mentioned mass spectra libraries and with those in the literature (Adams 2007). The
151 linear retention index of each component was determined adding a C_8-C_{32} n-alkanes
152 (Sigma-Aldrich) to the EO before injecting in the GC-MS equipment and analyzed
153 under the same conditions reported above (Guerrini et al. 2014)[14].

154

155 **Preparation of mother solution for biological assays**

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

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6 161 **Cell culture and virus**
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9 162 African green monkey kidney (Vero) cells were grown at 37° C in a humidified
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11 163 atmosphere, with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Lonza,
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13 164 Milan, Italy) supplemented with 10% inactivated fetal calf serum (FCS, Flow
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15 165 Laboratories, Irvine, UK.), 2 mM glutamine, 2% non-essential amino acids (Gibco,
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17 166 Paisley, UK), penicillin (100 IU/mL), and streptomycin (100 μg/mL). WNV strain,
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19 167 lineage 1, isolated from a patient during the WNV outbreak occurred in Sardinia Region
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21 168 in 2011 (Magurano et al. 2012) [15] was used for the study. For virus production
22
23 169 monolayers of Vero cells in 75-cm^2 tissue culture flasks were infected with WNV. After
24
25 170 5 days at 37° C, infected cells were harvested with freeze-and-thaw cycle, and cellular
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27 171 debris was removed with low-speed centrifugation, and virus titer was measured by
28
29 172 standard plaque assay. The virus was stored at −80° C until used.
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37 174 **Plaque assay**
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40 175 WNV titer was determined by plaque assays in Vero cells growing in 24-well plates.
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42 176 Briefly, serial tenfold dilutions of the viral suspension were added (0.1 mL/well) in
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44 177 duplicate. After infection, the cells were incubated for 1 h at 37°C. Subsequently,
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46 178 Tragacanth gum powder (SIGMA cat. G1128-100G) supplemented 1:1 with DMEM
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48 179 medium with 5% inactivated FBS, 2 mM glutamine, 2% non-essential amino acids,
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50 180 penicillin (100 IU/mL), and streptomycin (100 μg/mL) was added, and the plate was
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52 181 incubated for 5 days at 37° C. The viral plaques were visualized by 1% crystal violet
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54 182 solution (Fortuna et al. 2015). The titer was estimated by counting the number of
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3 183 plaques observed in each well and expressed as plaque-formation unit per milliliter
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5 184 (p.f.u./mL) (viral titer: 6,93x10^{6} p.f.u./mL).
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11 186 **Cytotoxicity assay**
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14 187 A cytotoxicity test was performed for all EOs. Each EO was diluted 1:10 in DMSO and
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16 188 subsequent several dilutions were made in MEM medium. Vero cell monolayers were
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18 189 put in contact with the mixture of MEM medium plus oil and maintained at 37° C with
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20 190 5% CO2. The cells were monitored daily to check the EO toxicity. After 24 h, the
21
22 191 following parameters were evaluated: cell morphology and viability (determined by
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24 192 neutral re[illegible]aining) were examined by light microscopy and cell proliferation was
25
26 193 evaluated quantitatively by microscopic counts after dispersion into individual cells
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28 194 with trypsin. EO dilutions that did not affect any of these parameters were considered as
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30 195 non-cytotoxic concentrations and utilized for antiviral assays. For neutral re[illegible]aining
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32 196 the 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduces
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34 197 the optical density (OD) of treated cells to 50% with respec[illegible] untreated cells
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36 198 (Pietrantoni et al. 2015).
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41 200 **Dose-Response Assay**
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44 201 The antiviral activity of *O. quixos* and *P. aduncum* EOs was assayed by neutral re[illegible]
45
46 202 assay. Briefly, in 96-well culture plate, monolayer cultures of Vero cells were incubated
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48 203 with different concentrations of *O. quixos* and *P. aduncum* starting from 2.6 μg/mL
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50 204 during the virus attachment step (1h 4° C). As viral inoculum was utilized WNV at a
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52 205 multiplicity of infection (m.o.i.) of 0.1 p.f.u./cell. After adsorption, Vero cells were
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rinsed thoroughly and incubated with the same concentrations of EO. The cells were maintained at 37° C in 5% CO_2. 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 (50 mg/mL), thereafter cells were washed with Phosphate Buffered Saline (PBS) and fixed with 4% formaldehyde, 10% $CaCl_2$. 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 (EC_{50}). The EC_{50} was calculated according to the following equation: $[(A – B) / (C – B) \times 100]$, 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 CC_{50} and EC_{50} data and applying the formula $SI = CC_{50}/EC_{50}$.

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 it 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

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incubated at 37°C in 5% CO_2 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% CO_2 for 1 h, the cells were washed and DMEM maintenance medium was added and the cells were maintained at 37° C in 5% CO_2.

ii. Cell monolayers were pre-treated 1h 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% CO_2 for 1h. Then MEM maintenance medium was added and the cells were maintained at 37° C in 5% CO_2.

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 neutr 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; $p \leq 0.05$) were calculated using software STATISTICA 6. (tatSoft Italia srl).

~~**Results**~~

~~**Chemical composition of EOs**~~

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~~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-ocimene (7.53%) and E-caryophyllene (4.80%) were the main compounds.~~

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~~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~~

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276 viral replication but *O. quixos* was more effective at concentration of 268 and 134,4
277 µg/ml respect *P. aduncum* that showed a viral inhibition similar for all concentration.
278 The 50% effective concentration (EC50) for *O.quixos* was 372 µg/ml whit a selectivity
279 index (SI) of 2.2 as showed on (Table 2).

280

281 **Antiviral activity of O. quixos on WNV**

282 In order to better investigate the inhibitory effects of *O. quixos* on WNV, EO was added
283 at different stages during viral infection. As showed on Fig 2 (A), pre-treatment of virus
284 with *O. quixos* showed relevant virucidal activity at concentrations of 33.6 µg/ml and
285 16.8 µg/ml. The EO was also effective at these concentrations when host cells were pre-
286 treated with drugs prior to infection, showing a percentage of cell vitality of 60% (Fig 2
287 (B)). Same results were obtained when *O. quixos* EO at concentration of 33.6 µg/ml and
288 16.8 µg/ml was present during the adsorption step (Fig 2 (C)). We, therefore, observed
289 similar inhibition effect on viral replication in all three conditions studied, when the EO
290 was used at the concentrations of 33.6 µg/ml and 16.8 µg/ml, with a percentage of cell
291 viability around 60%. No relevant inhibition was observed when the WNV was pre-
292 treated with EO at the concentrations of 2.1 µg/ml and 1 µg/ml, for all conditions used
293 (Fig 2 (A,B,C)).

294

295 **Antiviral activity of *P. aduncum* on WNV**

296 As showed on Fig 3 (A) antiviral activity of the *P. aduncum* was most pronounced
297 when viruses were treated before inoculation showing a percentage of cell viability of
298 79% at the concentrations of 33.6 µg/ml, 88% at 16.8 µg/ml, 87% and 78% at 2.1 an 1
299 µg/ml, respectively. When cells were incubated with EO only 1 µg/ml showed a small
300 protection (68% of cell viability) while no relevant antiviral activity was showed by the

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301 other concentrations (Fig 3(B)). When the EO was added during infection, the results
302 showed an antiviral activity at all the concentrations with a percentage of cell viability
303 of around 68% at 1 μg/ml (Fig 3 (C)).
304

305 **Discussion and conclusion**

306 Viral diseases are still a major problem for human health worldwide. Although natural
307 products have inherently high chemical diversity, plant-based products or bioactive pure
308 compounds obtained from EOs may be a new source of antiviral drugs but a few
309 number of studies focus on this research field. So far, only a limited number of drugs
310 are effective against many of these viruses, which has prompted research into finding
311 new antiviral lead molecules (Li et al. 2013, Elizaquível et al. 2013, Tanu and Harper
312 2016, Schnitzler et al. 2007, Astani et al. 2010, Lohézic-Le Dévéhat et al. 2002,
313 Ocazionez et al. 2010, Swamy et al. 2016). *P. aduncum* EO has been investigated by
314 several authors mainly focusing chemical characterization, antimicrobial, insecticidal,
315 larvicidal and anti-protozoic (Guerrini et al. 2009, Bernuci et al. 2016, Oliveira et al.
316 2013, Villamizar et al. 2017, Ling A et al. 2009, Monzote et al. 2017), but no data are
317 available regarding antiviral activity. Also *O. quixos* EO was characterized and tested
318 for its antimicrobial, antiplatelet and antithrombotic activity (Sacchetti et al. 2006,
319 Naranjo 1981, Rolli et al. 2014, Tognolini et al. 2006, Ballabeni et al. 2007); from our
320 knowledge no antiviral activities test were performed until now. The limited efficacy of
321 the current treatment of WNV infection enhances the need for novel therapies that
322 include substances with innovative viral targets and/or mechanisms of action. Our study
323 has been performed to analyse the potential capacity of *O. quixos* and *P. aduncum* EOs,
324 collected in the Ecuadorian Amazon, to reduce the WNV replication in infected cells.

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3 325 The chemical composition of leaf *O. quixos* EO, showing 1,8-cineole (39.15%) as main
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5 326 component, cinnamate derivatives and E-caryophyllene among minor compounds, did
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7 327 not reflect our previously results (Sacchetti el al. 2006), where E-caryophyllene,
8
9 328 cinnamyl acetate and other derivatives were the characteristic molecules. The yield was
10
11 329 instead comparable with our previous results. However, it should be noted that the
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13 330 variation of chemical composition for this EO has not yet been studied extensively. *P.*
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15 331 *aduncum* EO showed an overlapping composition to our previously data (Guerrini et al.
16
17 332 2009), with small quantitative differences regarding minor compounds: furthermore,
18
19 333 germacrene D (3.05%) was not detected in the previous studies. *P. aduncum* EO have
20
21 334 been largely studied in the last two decades and the formation of two chemotypes by
22
23 335 different biosynthetic routes has been evidenced. In fact, according to our data, Maia et
24
25 336 al. (1998), Cicció and Ballestero (1997), Fazolin et al. (2007), De Almeida et al. (2009)
26
27 337 isolated EOs from leaves of *P. aduncum* in different localities of Amazonian region and
28
29 338 determined that dillapiole, formed by the shikimate pathway, was the main compound
30
31 339 with a variability from 31.5% to 97.3%. The study of *P. aduncum* cultivation in
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33 340 Western Amazonian region confirmed dillapiole as major component (Silva et al. 2014).
34
35 341 If the leaves were instead collected from species in Atlantic Forest, and Northeastern
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37 342 and Southeastern Brazil, terpene compounds such as (E)-nerolidol and linalool were
38
39 343 detected as main components (De Almeida et al. 2009, De Oliveira et al. 2006,
40
41 344 Navickiene et al. 2006). The yield of dillapiole-chemotype EO in literature ranged from
42
43 345 0.35% to 4.0% (Guerrini et al. 2009, Fazolin et al. 2007, De Almeida et al. 2009, Silva
44
45 346 et al. 2014, Rali et al. 2007), our results instead showed a lower level (0.16%)
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47 347
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49 348 EOs were tested for their cytotoxicity on Vero cells, prior to the determination of their
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51 349 inhibitory effect against WNV. *P. aduncum* resulted relatively more toxic than *O.*
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3 350 *quixos*. The potential antiviral effect of different EOs was determined against WNV on
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5 351 Vero cells in vitro and both EOs tested exhibited a reduction of infectivity at non-
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7 352 cytotoxic concentration.
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9 353 High antiviral activity was observed for selected EOs when WNV was incubated whit
10
11 354 this substances prior host cell infection (virucidal activities). WNV is an enveloped
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13 355 virus and the results of our study highlighted that both *O. quixos* and *P. aduncum* show
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15 356 virucidal activity.
16
17 357 Experiments aimed to assess the antiviral activity of EOs have been most frequently
18
19 358 conducted on viruses of the herpes group, enveloped viruses (Novak 2011). Schnitzler
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21 359 et al. (2007) demonstrated a virucidal effect of peppermint oil, when herpes simplex
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23 360 virus was mixed with the essential oil prior to inoculation. The application of tea tree
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25 361 oil, the EO of Melaleuca alternifolia, for the treatment of recurrent herpes labialis has
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27 362 been recently reported (Carson et al. 2001, Schuhmacher et al. 2003). Accordingly, a
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29 363 virus lacking of envelope, like adenovirus, was not affected by eucalyptus EO (Cemelli
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31 364 et al. 2008). Therefore our results suggest that *O. quixos* and *P. aduncum* could directly
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33 365 inactivate WNV and might interfere with virion envelope structures or mask viral
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35 366 structures which are necessary for adsorption or entry into host cells.
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37 367 In this study, experiments were also performed to determine the viral inhibitory effect
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39 368 treating the cells with EOs before adsorption. This different approach was important to
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41 369 better investigate on mechanisms of antiviral action of the EOs. In our study, *O. quixos*
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43 370 showed a protection of the cells from viral infection unlike *P. aduncum*. These findings
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45 371 would suggest an ability of *O. quixos* to inhibit viral replication by interfering with the
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47 372 virus binding to the cells. *P. aduncum* didn't result protective when added to the cells
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49 373 before inoculum. In this case we might, in some ways, assume that the oil does not act
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374 by competing with the virus for binding to the cell, and that part of its activity relies on
375 direct inactivation of the viral particles after virus adsorption.
376 For *O. quixos* we observed a good and similar protection from WNV infection adding
377 the EO to the DMEM medium during the intracellular replication period, up to an EO
378 concentration of 16.8 µg/ml. These results suggest that *O. quixos* could act directly on
379 WNV virus and might interfere with virion envelope structures or mask viral structures,
380 which are necessary for adsorption or entry into host cells. Thus different mechanisms
381 of antiviral activity of different EOs and compounds seem to be present.
382 All together, these results support the potential use of EOs *in toto* from medicinal plants
383 as agents for the treatment of viral infections. The effectiveness of the EOs from
384 Amazonia against the viruses tested was variable, but their virucidal properties against
385 these viruses suggest the application of this type of natural products as disinfectants or
386 topical medicaments.
387 Previous studies with EOs from eucalyptus, tea tree and thyme (Astani et al. 2010),
388 have shown the direct inactivating action of EOs and their components on virion
389 infectivity (HSV-1), with the exception of 1,8-cineole. In particular, α-pinene, α-
390 terpineol, terpinen-4-ol and p-cymene, detected in considerable amount in O. quixos
391 EO, revealed a high antiviral activity and could be responsible for inactivation action.
392 However, in our research, further investigation is required to better elucidate the active
393 components and their mixture responsible of the inhibitory effect on virions.
394

395 **Results and Discussion**

396 The limited efficacy of the current treatment of WNV infection enhances the need for
397 novel therapies that include substances with innovative viral targets and/or mechanisms
398 of action. So far, only a limited number of plant-derived products are effective against

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399 viruses, which has prompted research into finding new antiviral lead molecules
400 (Lohézic-Le Dévéhat et al. 2002; Schnitzler et al. 2007; Astani et al. 2010; Ocazionez et
401 al. 2010; Elizaquível et al. 2013; Li et al. 2013; Swamy et al. 2016; Tanu and Harper
402 2016).

403 Our study has been performed to analyze the potential capacity of *O. quixos* and *P.*
404 *aduncum* EOs, collected in the Ecuadorian Amazon, to reduce the WNV replication in
405 infected cells. *O. quixos* EO was characterized and tested for its antimicrobial,
406 antiplatelet and antithrombotic activity (Naranjo 1981; Sacchetti et al. 2006; Tognolini
407 et al. 2006; Ballabeni et al. 2007; Rolli et al. 2014); from our knowledge no antiviral
408 activities test were performed until now. *P. aduncum* EO has been investigated by
409 several authors mainly focusing on chemical characterization, antimicrobial,
410 insecticidal, larvicidal and anti-protozoic activities (Guerrini et al. 2009; Ling et al.
411 2009; Oliveira et al. 2013; Bernuci et al. 2016; Monzote et al. 2017; Villamizar et al.
412 2017). In addition, *P. aduncum* was found active on Poliovirus (Lohézic-Le Dévéhat et
413 al. 2002).

414 In the present work the chemical composition of *O. quixos* and *P. aduncum* EOs was
415 determined by GC-MS and GC-FID. The yield of distillation for *O. quixos* EO was
416 0.13±0.01% (w/v), comparable with our previous results (Sacchetti el al. 2006). The
417 main component was represented by 1,8-cineole (39.15%), followed in less amount by
418 α-terpineol (7.65%), sabinene (6.46%), α-pinene (6.27%), p-cymene (6.12%), E-
419 caryophyllene (4.73%), terpinen-4-ol (4.22%) (Table 1): monoterpenes were therefore
420 the main fraction of EO. This chemical profile did not reflect our previous data
421 (Sacchetti el al. 2006), where E-caryophyllene, cinnamyl acetate and other derivatives
422 were the characteristic molecules. Moreover, methyl cinnamate, a typical
423 phenylpropanoid of floral calyx EO (Bruni et al. 2004), was a minor compound derived

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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 phenylpropanoid 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%).

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448 EOs were tested for their cytotoxicity on Vero cells prior to the determination of their
449 inhibitory effect against WNV, by using a neutral red assay in which the half maximal
450 cytotoxic concentration (CC_{50}) value of each compound was calculated. *P. aduncum*
451 resulted relatively more toxic than *O. quixos*. Indeed, the results illustrated a CC_{50} value
452 of 163 μg/mL for *P. aduncum* and 840 μg/mL for *O. quixos*. Treated cells with vehicle
453 control, 1% DMSO, did not show any cytotoxicity against Vero cells (Table 2).
454 In order to evaluate the effect of *O. quixos* and *P. aduncum* on viral replication, Vero
455 cells were infected with WNV at 0.1 m.o.i. p.f.u./cell and exposed to various two-folds
456 concentrations of EO starting from 2.6 μg/mL for 72 h post-infection (p.i.).
457 Results are presented in Figure 1 as percentage of cell viability and represent the
458 average of three independent experiments. As shown in the figure, both EOs were able
459 to inhibit viral replication but *O. quixos* was more effective at concentration of 268 and
460 134.4 μg/mL respect *P. aduncum* that showed a viral inhibition similar for all
461 concentrations. The 50% effective concentration (EC_{50}) for *O. quixos* was 372 μg/mL
462 with a selectivity index (SI) of 2.2 (Table 2).
463 In order to better investigate the inhibitory effects of *O. quixos* and *P. aduncum* on
464 WNV, EOs was added at different stages during viral infection. As shown on Figure 2,
465 no relevant differences between the antiviral activities ~~of OE~~ of *O. quixos* OE were
466 observed in all conditions studied. The highest percentages of cell viability (>60%)
467 were observed when the EO was added to the host cells at the concentrations of 16.8
468 and 33.6 μg/mL prior to the infection. Similar results were obtained when 33.6 μg/mL
469 of EO were added during the adsorption step. Differently, when the EO was added at
470 low concentrations (1 and 2.1 μg/mL) no relevant antiviral activity was observed in any
471 conditions.

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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 manly 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

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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*.

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.

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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 of 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).

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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~~

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~~significance (Student's t test; $p \leq 0.05$) were calculated using software STATISTICA 6.0 (StatSoft Italia srl)~~

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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 ($p<0.05$)

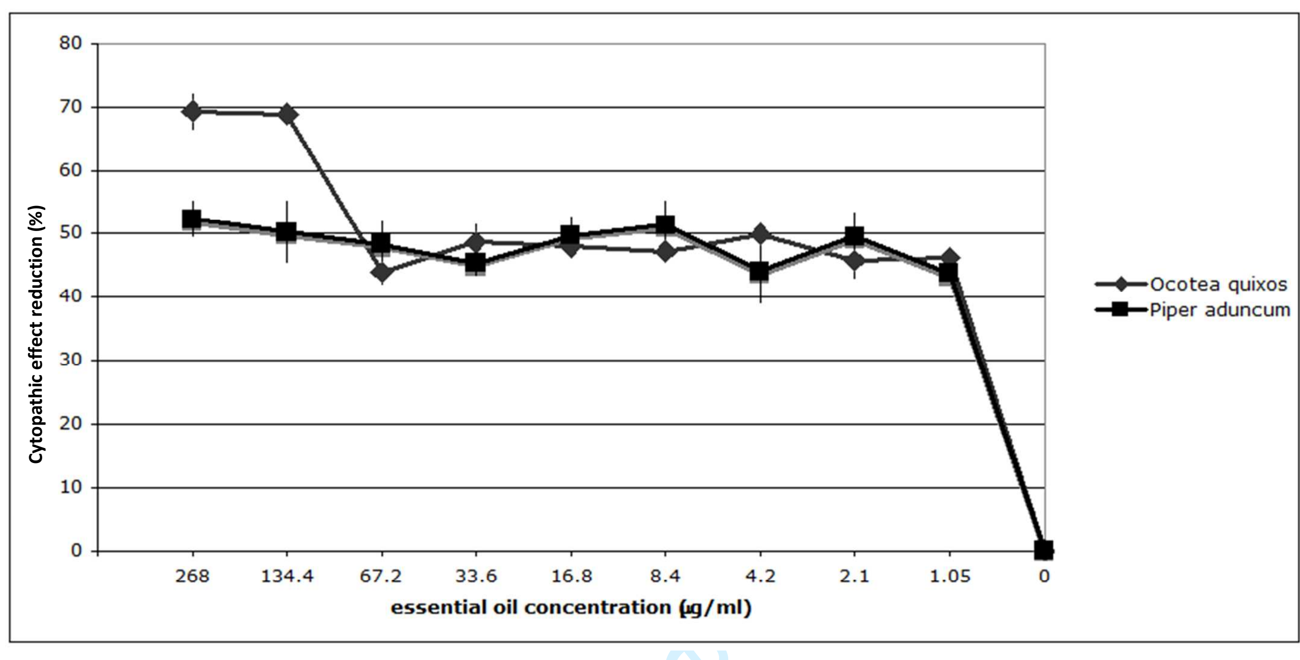

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Figure 2. Antiviral activity of *O. quixos* against WNV at 0.1 m.o.i.p.f.u./cell (**A**) after incubation of virus with different concentration of EO 1h at 37° C before infection ; (**B**) after pre-treatment of cells with drugs before viral infection 1h at 37° C (**C**) after treatment with different concentration of EO during infection. CC = cell control; CV = virus control

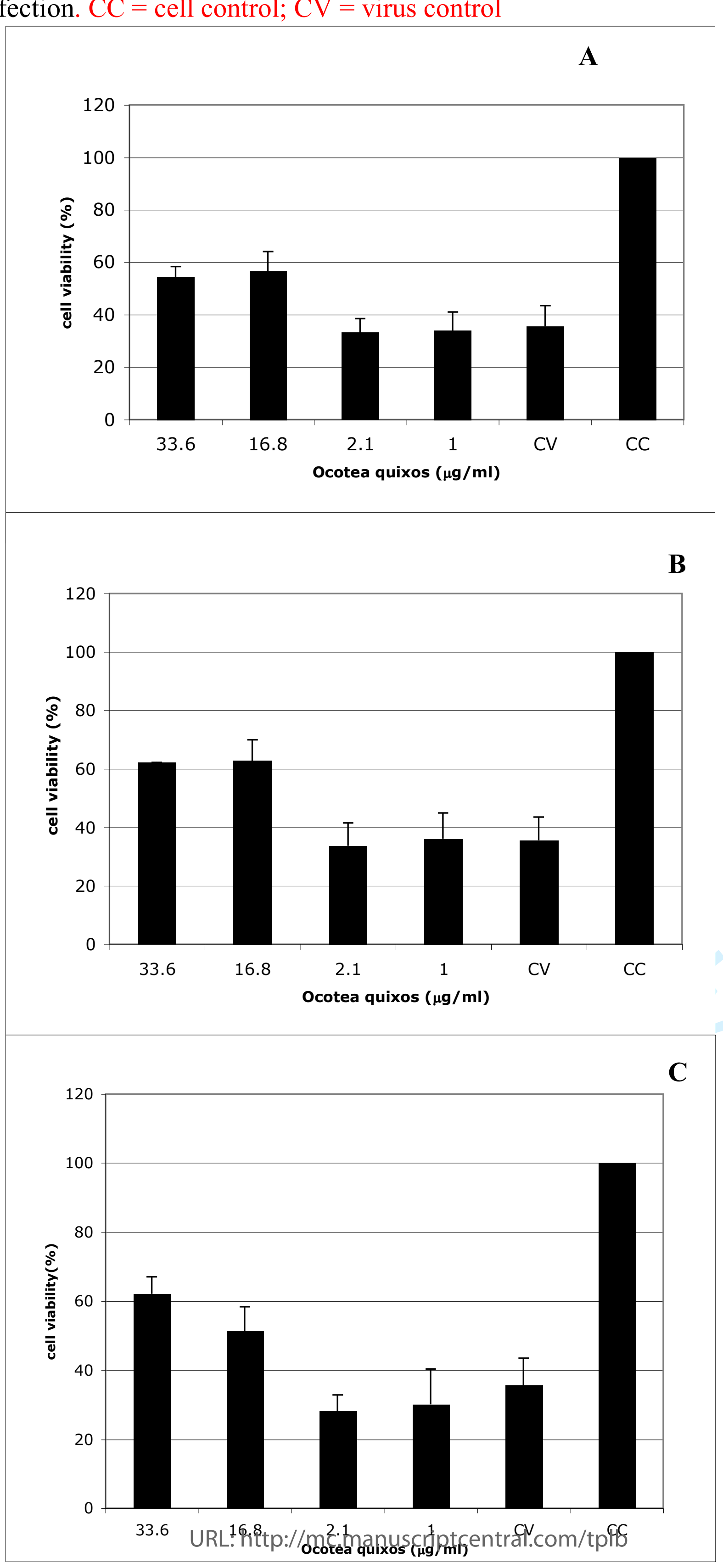

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Figure 3. Antiviral activity of *P. aduncum* against WNV at 0.1 m.o.i. p.f.u./cell (**A**) after incubation of virus with different concentration of EO 1 h at 37° C before infection; (**B**) after pre-treatment of cells with drugs before viral infection 1 h at 37° C (**C**) after treatment with different concentration of EO during infection. CC = cell control; CV = virus control

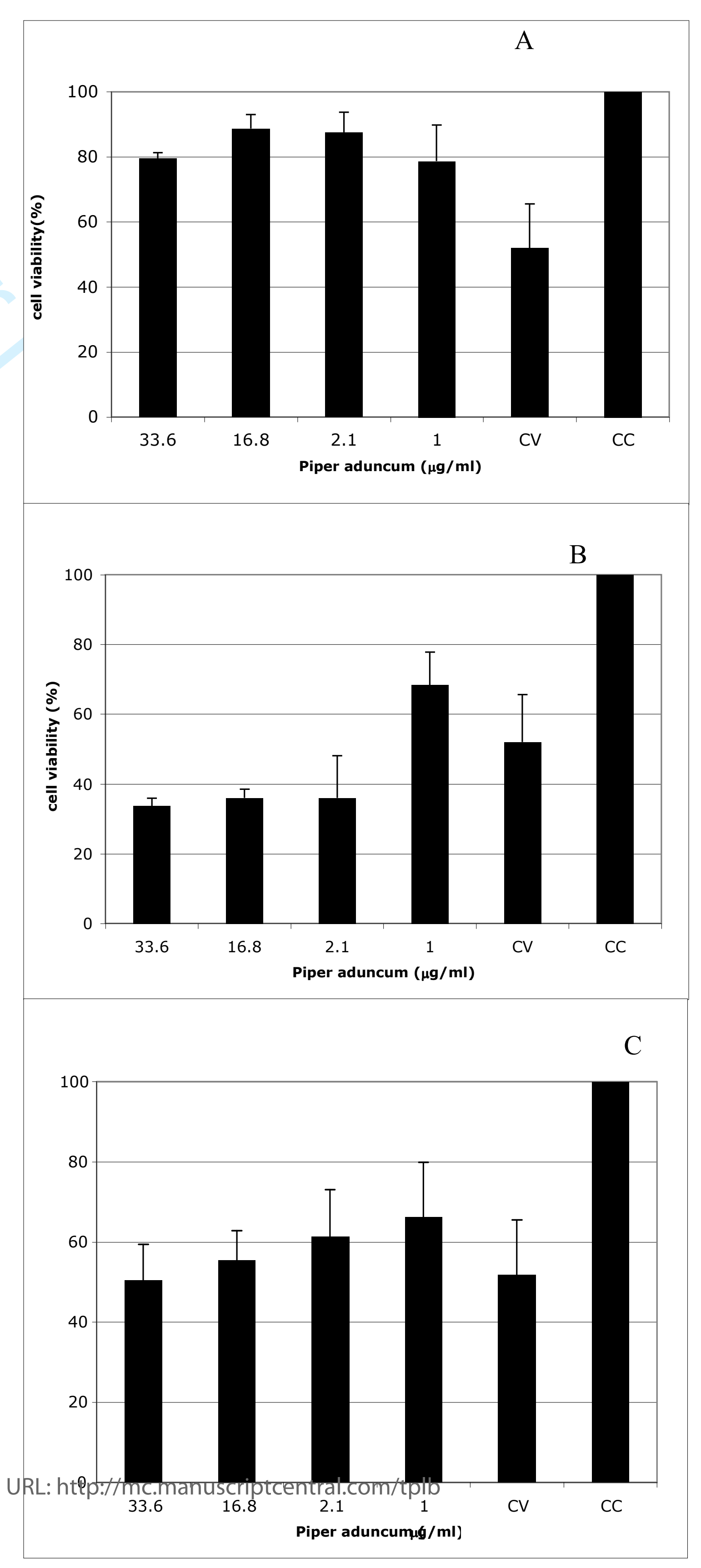

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Table 1. Chemical composition of *O. quixos* and *P. aduncum* EOs

No.	**Component**[1]	***O. quixos* (Area %)**[2~~1~~]	***P. aduncum* (Area%)**[2~~1~~]	**RI exp**[3~~2~~]	**RI lit**[4] ~~**lett**~~[~~3~~]
1	α-thujene	1.50±0.09	0.23±0.02	922	**924**
2	**α-pinene**	**6.27**±0.47	1.63±0.14	929	**932**
3	camphene	0.16±0.02	-	944	**946**
4	**sabinene**	**6.46**±0.39	-	967	**969**
5	β-pinene	3.45±0.21	0.99±0.08	973	**974**
6	myrcene	0.83±0.06	0.42±0.03	987	**988**
7	α-phellandrene	0.31±0.03	0.78±0.05	1005	**1002**
8	p-mentha-1(7),8-diene	0.48±0.04	0.16±0.01	1006	**1005**
9	α-terpinene	1.73±0.11	0.63±0.04	1014	**1014**
10	**p-cymene**	**6.12**±0.42	1.48±0.13	1021	**1020**
11	o-cymene	-	1.21±0.10	1025	**1023**
12	limonene	1.84±0.11	1.10±0.11	1026	**1024**
13	**1,8-cineole**	**39.15**±2.32	-	1028	**1026**
14	cis-ocimene	-	3.33±0.27	1032	**1032**
15	***trans*-ocimene**	**-**	**7.53**±0.48	1043	**1044**
16	γ-terpinene	3.05±0.18	1.99±0.16	1053	**1054**
17	p-mentha-2,4(8)-diene	0.35±0.02	1.27±0.10	1082	**1085**
18	δ-terpineol	0.47±0.04	-	1167	**1162**
19	terpinen-4-ol	4.22±0.25	1.63±0.12	1176	**1174**
20	**α-terpineol**	**7.65**±0.51	-	1193	**1186**
21	piperitone	-	3.78±0.25	1250	**1249**
22	δ-elemene	-	0.10±0.01	1337	**1335**

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23	α-cubebene	0.40±0.03	0.18±0.02	1351	**1345**
24	cyclosativene	-	0.36±0.03	1369	**1370**
25	α-ylangene	-	0.18±0.01	1371	**1373**
26	α-copaene	1.44±0.08	1.23±0.11	1376	**1374**
27	β-cubebene	-	0.11±0.01	1387	**1387**
28	β-elemene	-	0.58±0.03	1388	**1389**
29	*trans*-methylcinnamate	1.53±0.06	-	1389	**1388**
30	α-gurjunene	-	0.50±0.02	1398	**1409**
31	**E-caryophyllene**	**4.73**±0.33	**4.80**±0.35	1410	**1416**
32	α-santalene	-	0.10±0.01	1414	**1417**
33	β-copaene	-	0.31±0.02	1424	**1430**
34	γ-elemene	-	0.18±0.01	1427	**1434**
35	aromadendrene	-	0.29±0.02	1432	**1439**
36	α-humulene	2.96±0.21	1.74±0.16	1451	**1452**
37	*trans*-cadina-1(6),4-diene	-	0.17±0.02	1470	**1475**
38	γ-muurolene	-	0.54±0.05	1473	**1478**
39	germacrene D	-	3.05±0.29	1477	**1484**
40	β-chamigrene	0.13±0.02	-	1480	**1480**
41	β-selinene	2.00±0.18	0.32±0.02	1484	**1489**
42	γ-amorphene	-	0.28±0.02	1487	**1495**
43	viridifilorene	-	0.52±0.04	1491	**1496**
44	bicyclogermacrene	0.79±0.06	-	1491	**1500**
45	α-muurolene	-	0.33±0.03	1495	**1500**
46	n-pentadecane	-	0.24±0.01	1500	**1500**

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47	(E,E)-α-farnesene	-	0.53±0.05	1504	**1506**
48	γ-cadinene	-	0.44±0.04	1509	**1513**
49	δ-amorphene	0.43±0.04	1.39±0.12	1516	**1511**
50	myristicin	-	0.64±0.05	1524	**1517**
51	*trans*-γ-bisabolene	0.26±0.02	-	1526	**1531**
52	*trans*-cadina-1(2),4-diene	-	0.16±0.01	1531	**1533**
53	α-cadinene	-	0.10±0.01	1535	**1537**
54	germacrene B	-	0.50±0.04	1557	**1559**
55	spathulenol	0.10±0.01	0.64±0.04	1577	**1577**
56	caryophyllene oxide	0.44±0.03	0.48±0.03	1581	**1582**
57	viridiflorol	-	1.13±0.11	1593	**1592**
58	humulene epoxide II	0.19±0.02	0.27±0.02	1609	**1608**
59	**dillapiole**	**-**	**48.21**±2.65	1622	**1622**
	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).

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Table 2. CC_{50} = 50% cytotoxic concentration (µg/mL); EC_{50} = 50% effective concentration (µg/mL); SI~~50~~ = selectivity index (CC_{50}/ EC_{50})

	CC_{50}	**EC_{50}**	**SI**
O. quixos	840 µg/mL	372 µg/mL	2.2
P. aduncum	163 µg/mL	163 µg/mL	1