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The antiviral effect of mollusk mucus on measles virus

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ABSTRACT

Measles is a viral disease highly contagious spread by respiratory transmission. Although infection can be controlled by vaccination, numerous cases of measles have been registered in many areas of the world, highlighting the need for additional interventions. Terrestrial gastropods exude mucus on their body surface when traveling, to protect the body from mechanical injury, desiccation or contact with harmful substances. The mucus of mollusks has been studied as a source of new natural compounds with diverse biological activities. In this study, the antiviral activity of the mucus of the land slug *P. boraceiensis* was demonstrated in vitro using Vero cells infected with measles virus. The crude sample and four fractions were tested in cultures infected with measles virus and the antiviral activity was assessed by the cytopathic effect in infected cell cultures as well as by immunofluorescence and qPCR. Fractions 39 and 50 of the mucus from *P. boraceiensis* were analyzed by HPLC-DAD-ESI-MS/MS and infrared spectroscopy. A mixture of polyunsaturated fatty acids was found in the two fractions. A reduction in the growth of the measles virus was observed, measured by qPCR, with a protection index of 80% in Vero cells infected with measles and treated with fraction 39. Fraction 39 exhibited the best antiviral action in vitro and high contents of hydroxy-tritriacontapentaenoic acid and hydroxy-pentatriacontapentaenoic acid were found in this fraction.

1. Introduction

Measles virus (MV) is one of the most infectious microorganisms known and continues to cause extensive morbidity and mortality worldwide. Despite vaccine availability and the initiative launched by WHO, UNICEF, and their partners to increase vaccine coverage, MV has not been eradicated and has caused 140,000 deaths globally as recently in 2010 (Simons et al., 2012), making it one of the top causes of death among the vaccine-preventable diseases. MV infection causes an acute febrile respiratory illness with skin rash and may cause acute, profound suppression of the immune system. The neurological sequelae of measles can occur within days to years after acute infection, often resulting in severe disability and death (Hosoya, 2006). Acute post-infectious encephalomyelitis occurs primarily in older children and adults during or shortly after acute infection. Subacute sclerosing panencephalitis (SSPE) is a late neurodegenerative complication associated with the persistent infection of brain cells (Bale, 2014).

Veronicellidae are hermaphroditic mollusks that can perform cross-fertilization/self-fertilization. *Phyllocaulis boraceiensis* has a long and single period of reproduction between July and September (Toledo-Piza et al., 2012, 2013). *P. boraceiensis* mucus is an exudate that acts as the animal's defense system. Terrestrial slugs and snails produce mucus which performs a variety of functions, including facilitating movement along the ground, communication and a non-specific, defensive response to physical or chemical irritation. This material is a mucoprotein that “in natura” appears highly viscous; it contains 600 μg/mL glucose, 6.9×10^{-5} mg/mL lipids and 1.15×10^{-4} mg/mL of protein and aminoacid residues (Toledo-Piza et al., 2012).

A study on the effect of *Phyllocaulis boraceiensis* mucus in the induction of fibroblast proliferation, demonstrated an increase of

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over 50% in the number of cells when compared to the control experiment (Toledo-Piza et al., 2013). This same material was also able to induce angiogenesis in co-cultured endothelial cells and fibroblasts (Toledo-Piza and Maria, 2013). The biological activity of this mucus was also shown by enhanced dermal healing in mice submitted to a dorsal incision and treated daily with 100 μL of *P. boraceiensis* mucus. A reduced wound closure time was observed in mice treated with anointment containing 0.012 μg/mL mucus. Tissue remodelling began after four days of treatment and showed redness, edema and bleeding until the 5th day post-surgery. Granulation and healing tissue appeared intensely from the 11th day (Toledo-Piza and Maria, 2014).

There are no specific therapies for acute complications of MV or for persistent MV infections. Therefore, the search for new antiviral compounds is important due to the specific needs of immune compromised people at risk for MV infection, who cannot be vaccinated or do not respond adequately to vaccines.

Many studies have shown the importance of invertebrates, with an emphasis on mollusks, in the search for new antiviral compounds. Leading in consideration, the importance of active compounds extracted from invertebrates, our research group proposed to study *Phyllocaulis boraceiensis* mucus as a source of bioactive molecules with antiviral activity in MV cultures.

2. Material and methods

2.1. Collection and solubilization of Phyllocaulis boraceiensis mucus

In this study, 20 specimens of the terrestrial slug *Phyllocaulis boraceiensis* were farmed in the vivarium of the Malacology Laboratory at the Butantan Institute, São Paulo. The Ethics Committee on Animal Use at the Butantan Institute (CEUAIB) declared that this study does not involve the creation and/or use of animals in the phylum Chordata (I-1133/13).

The animals were kept in plastic boxes containing soil "in natura" and a screened cover, in a pollution-free laboratory environment with the temperature controlled at 24 °C with 85% relative humidity. All specimens were fed every two days with small amounts of lettuce (A. R. Toledo-Piza et al., 2013) and the boxes were cleaned every two days.

Before the collection of mucus, the animals were transferred to a large Petri dish and nebulized with distilled water (Li and Graham, 2007). The removal of the mucus was stimulated by mechanical processes that did not cause death or damage to the specimens (Pakarinen, 1994). The animals were cleaned for 5 min on the smooth surface of the Petri dish containing a thin layer of saline solution (0.06% NaCl). The addition of this solution to the collection plates facilitated the release and removal of mucus, which was removed with the aid of a spatula and transferred to a storage container. The solubilization/dissolution process required organic solvents, such as ethanol and methanol (A. R. Toledo-Piza et al., 2013).

An amount of 20 mL of crude mucus were collected. After solubilization process and lyophilization an amount of 500 μg of crude mucus in pounder were tested. The *P. boraceiensis* mucus samples were concentrated using a membrane of regenerated cellulose (Ultracel®-3H-AmiconUltra, Millipore). The supernatant obtained after filtration was cleaning using a "2D Clean Up Kit" (GE Healthcare). This solution was used in all experiments.

2.2. Fractionation of Phyllocaulis boraceiensis mucus

Stored samples of *P. boraceiensis* mucus were centrifuged at 1,000*g* for 10 min and filtered through a sterilizing 0.22 μm membrane. For the fractionation of the mucus, a gel filtration column was used (Superdex™ 75, GE Healthcare) coupled to a high pressure chromatograph (Akta Purifier, GE Health care). The flow rate was kept constant at 1.0 mL min^{-1} and the mobile phase consisted of two solvents: eluent A (0.1% aq. formic acid in Milli-Q water) and eluent B (methanol 0.1% aq. formic acid).

Analysis of fractions 39 and 50 of *Phyllocaulis boraceiensis* mucus using Fourier transform infrared spectrometry (FT-IR).

FT-IR spectra were recorded using a Bomem spectrometer by scanning over the frequency range of 4000–400 cm^{-1} at a resolution of 5 cm^{-1}. Fractions 39 and 50 were dissolved in methanol and analyzed using potassium bromide pellets.

Analysis of fractions 39 and 50 of *Phyllocaulis boraceiensis* mucus using reversed phase high performance liquid chromatography-diodo array-electrospray ionization mass spectrometry/mass spectra (HPLC-DAD-ESI-MS/MS).

HPLC-DAD-ESI-MS/MS analysis was conducted on a DADSPD-M10AVP Shimadzu system equipped with a photodiode array detector coupled to an Esquire 3000 Plus system (Bruker Daltonics), which consisted of two LC-20AD pumps, an SPD-20A diode array detector, a CTO-20A column oven and an SIL 20AC auto injector (Shimadzu Corporation Kyoto, Japan). The mass detector was a quadrupole ion trap equipped with an atmospheric pressure ionization source through an electrospray ionization interface, which was operated in full scan MS/MS mode. All the operations, acquisition and data analysis were controlled by CBM-20A software. The mobile phase was filtered through a 0.45 mm Millipore filter and degassed prior to use. Fractions 39 and 50 were dissolved in methanol:water (80:20 v/v) and filtered through a 0.45 μm PTFE filter, prior to injecting 50 μL into the HPLC system. The peaks were also monitored by diode array detection at a wavelength of 270 nm. The mobile phase consisted of two solvents: eluent A (0.1% aq. formic acid in Milli-Q water) and eluent B (methanol 0.1% aq. formic acid). Constituents were separated using a reverse phase, Phenomenex Gemini C-18 (250 × 4.6 mm, 5 μm) column connected to a guard column. The elution started with 10% B in A; 5 min – 20% B in A; 15 min – 30% B in A; 20 min – 40% B in A; 25 min – 50% B in A; 30 min –60% B in A, 35 min – 70% B in A; 40 min – 80% B in A; 50 min – 100% B in A and finally back to the initial conditions (10% B in A) to re-equilibrate the column prior to another run. The flow rate was kept constant at 1.0 mL min^{-1} and the temperature of the column was maintained at 40 °C. The ionization conditions were adjusted as follows: electrospray ionization was performed using an ion source voltage of 38 V and a capillary offset voltage of 4500 V. The full scan mass acquisition was performed using electrospray ionization in positive ion mode by scanning from m/z 100–1200 mass units. Helium was used as the collision gas and nitrogen as the nebulizing gas. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 27 psi. Desolvation was enhanced using a counter current nitrogen flow set at a flux of 7.0 L/min and a capillary temperature of 320 °C. The data dependent MS/MS events were assessed based on the most intense ions detected in full scan MS. The maximum accumulation time of the ion trap and the MS numbers to obtain the MS average spectra were set at 30 and 3 MS, respectively. All peaks were defined through the interpretation of ESI-MS and ESI-MS/MS spectra and by comparison with literature data (Murphy, 2014).

2.3. Cells and viruses

Vero cell lines (African green monkey kidney, ATCC CCL-81) were cultured in 75 cm^2 plastic cell culture flasks using L-15 medium supplemented with 10% inactive fetal bovine serum (FBS) and 20 mM L-glutamine (Invitrogen, EUA). The MV Edmonston wild-type strain used in this study has been previously described (Rota et al., 1994) and was used to assess the antiviral activity of the

P. boraceiensis mucus and its fractions.

2.4. MTT assay

The evaluation of the toxicity of *P. boraceiensis* mucus was performed using the MTT assay, whereby cell viability is assessed by mitochondrial activity and evaluated by the activity of the enzymes sorbitol dehydrogenase (SDH) and lactate dehydrogenase (LDH), which reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals (Mosmann, 1983). Vero cells were cultured in 96-well plates in L-15 medium supplemented with 10% fetal bovine serum. After 48 h, cells were treated with either the crude mucus or its fractions at a concentration of 0.5 mg/mL in the culture medium. After 24 h of exposure, the supernatant was discarded and MTT was added at a concentration of 500 μg/mL and incubated for 4 h. The culture medium was removed and 100 μL of dimethyl sulfoxide was added. The plates were shaken for 30 min and then read on a spectrophotometer at 570 nm.

2.5. Determination of the virus infectious dose

Confluent Vero cell monolayers were dispersed with 0.2% trypsin and 0.02% versene and solubilized in L-15 growth medium with 100 IU/mL penicillin G and 100 mg/mL streptomycin. For the preparation of 96 well plates, the cell suspension was diluted to 2.0×10^4 cells/mL. Plates were seeded with 200 μL of the suspension and incubated at 37 °C. The MV strain (Edmonston) stock virus was quantified by tissue culture infection with a 0.01 MOI (multiplicity of infection). The confluent cell cultures were inoculated with 100 μL of diluted virus in quadruplicate. After 1 h of adsorption at 37 °C, each well received 200 μL of L-15 medium with 10% FBS. Uninfected cultures were also prepared and treated identically as controls. Cultures were assessed for the cytopathic effect (CPE) daily for seven days, when the test was concluded. Fifty per cent infectivity end points were calculated by the method of Reed (Reed and Muench, 1938). All titers are given as log 10 $TCID_{50}$ per 0.1 mL of virus.

2.6. Effect of Phyllocaulis boraceiensis mucus on measles viral replication

The crude sample of mucus and its chromatographic fractions were tested in Vero cells infected with MV. For this study, Vero cells were pretreated (60 min) with 2%/100 μL of mucus or its fractions. After this period, the cells were infected with 1 MOI of viral particles per cell of MV. These cultures were kept at 37 °C for 3, 5 or 7 days. The antiviral effect of mucus and its fractions was determined through an optical microscope and the evaluation of the CPE. After this, the CPE was determined as the highest dilution of virus capable of inducing the CPE in infected cultures treated with mucus. The intensity of the antiviral effect was determined by the observed differences in the CPE between infected cultures and infected cultures treated with mucus.

In vitro antiviral assay using the mucus and fractions 39, 40, 49 and 50.

The potential antiviral activity of *P. boraceiensis* mucus and fractions 39, 40, 49 and 50 were assayed. Cell monolayers were incubated with 60, 120 and 180 ng/mL of *P. boraceiensis* mucus and fractions 39, 40, 49 and 50 for 1 h at 37 °C, followed by washing with L-15 and incubation with MV (1 MOI). After washing off the virus, cells were incubated for a further 72 h at 37 °C. After this, the determination of the effect of the mucus and mucus fractions on the infected cells was carried out using real-time qPCR.

2.7. Quantitative real-time PCR (qPCR)

Total RNA was extracted from 200 μL of infected and non-infected cultures using MagNA Pure Extractor (Roche, Basel, Switzerland). The assay was performed in triplicate in 25 μL reaction volume containing reaction buffer (Invitrogen, Carlsbad, CA, USA), 0.5 μL of a Superscript-Taq enzyme mixture, 0.2 μM of each primer, 0.1 μM of the labeled probe (Invitrogen, Carlsbad, CA, USA) and 5 μL of RNA. Thermal cycling was carried out on an Applied Biosystems 7500 apparatus at 50 °C for 10 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min (Hummel et al., 2006). The presence of intact RNA in the samples was confirmed with specific primers to RNAseP (Emery et al., 2004). Known copy numbers of transcribed MV RNA (four positive controls) were run for quantification purposes. Standard curves were prepared by qPCR using serial dilution with known copy numbers of the purified amplification product for MV. The copy number of the samples was calculated from the standard curves (Hummel et al., 2006). All experiments were performed in triplicate and pure dimethyl sulfoxide was added to cells infected with MV (1 MOI) as a control.

2.8. Determination of antiviral activity against infected cell cultures using fluorescent antibodies

Visualization of viral infection by MV was performed by immunofluorescence assays using a specific monoclonal antibody. Vero cells were cultured in 96-well microplates and infected with 1 MOI of virus, when the cell culture was semi-confluent. After 24 h, the cells were rinsed twice with PBS and fixed in 4% paraformaldehyde (Fluka) for 20 min. After fixation, the cells were washed three times with PBS, permeabilized with Triton X-100 0.1% (Sigma) for 10 min and blocked with PBS, 1% BSA (Sigma) and 50 μg/mL RNAase A (Invitrogen) at 37 °C for 1 h. After this, the cells were incubated with 10 μg/mL of mouse monoclonal anti-measles FITC antibody (Millipore) in 50 μL of PBS+1%BSA solution at 4 °C overnight. The cells were washed three times with PBS containing 0.05% Tween 20 (Sigma) before incubations with 20 μg/mL propidium iodide (Sigma) in 50 μL of PBS + 1% BSA for 1 h. After this incubation, the cells were washed five times with PBS + Tween 20 and 50 μL of an anti-fading solution with PBS, 50% glycerol (GE Healthcare) and ρ-phenylenediamine 0.1% (Sigma) was added to each well. The images were acquired with an Axio CamMRc attached to a Zeiss Axio Vert. A1 microscope using a LD Plan-NEOFLUAR 20x/0.4.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, USA), and the statistical significance of differences between groups was determined using unpaired one-way ANOVA. For the analysis of cell viability data and antiviral activity for the quantification of mRNA, Student's t-test was used with the p-value corrected by the Bonferroni-Sidak test. Comparisons were considered significant at $\rho < 0.05$.

3. Results

3.1. Fractionation of Phyllocaulis boraceiensis mucus

The chromatographic profile of *Phyllocaulis boraceiensis* mucus obtained from the Akta Purifier high pressure chromatograph and Superdex gel filtration column 75 is shown in Fig. 1, indicating the presence of four fractions. Biological experiments carried out using these four fractions showed that fractions 39 and 50 were the most active against MV.

Fractions 39 and 50 were analyzed using Fourier transform Infrared spectrometry (FT-IR) and reversed phase high performance liquid chromatography-diodo array-electrospray ionization mass spectrometry/mass spectra (HPLC-DAD-ESI-MS/MS).

The Fourier transform Infrared spectrum of fraction 39 showed absorption bands at 3368, 3205, 3010, 2955, 2917, 2849, 1629, 1517, 1463, 1402, 1378, 1296, 1059 and 1038 cm^{-1}. The large and strong band at 3368-3205 cm^{-1} indicated the presence of OH and COOH groups; the strong bands at 2599, 2917 and 2849 cm^{-1} (C–H, asymmetric and symmetric stretching vibration, respectively) indicated the presence of long carbon chains. The ratio of the peak area CH = CH (3010–2950 cm^{-1}) versus CH_2 (2955–2917 cm^{-1}) denoted the unsaturation index of fatty acids (Wu and He, 2014). A strong peak at 3010 cm^{-1} is originated from C–H stretching modes. The band at 1629 cm^{-1} was attributed to C=O stretching vibrations conjugated with double bonds (C=C) adjacent to a carbonyl group. The bands at 1517 and 1463 cm^{-1} were attributed to stretching of double bonds and CH_2 bending, respectively; the strong bands at 1059 and 1038 cm^{-1} were attributed to stretching vibrations of C–O and C–H bending. The C–O stretching vibrations in alcohols produce a strong band in the 1260-1000 cm^{-1} region of the spectrum (Shapaval et al., 2014). Changes in vibrational frequencies generally are a result of a number of different intermolecular and intramolecular interactions, such as steric effects or the formation of hydrogen bonds, which may influence the strength or angles of existing chemical bonds and eventually may change the vibrational frequencies of the corresponding groups (Kiefer et al., 2010; Shapaval et al., 2014). Generally, when a bond is strengthened it becomes more rigid, what increase the vibration frequency (Kiefer et al., 2010). For fraction 50, the main absorption band was detected at 1731 cm^{-1}, attributed to stretching of C=O groups of ketones or esters (Shapaval et al., 2014; Wu and He, 2014). Therefore, the main difference among the IR spectra of fractions 39 and 50 was the presence of band at 1731 cm^{-1} assigned to the C = O stretch, the bands detected in fraction 39 exhibited minor intensity than in fraction 50.

Peptides were found in mucus of mollusks (Pitt et al., 2015). However, in IR spectra of these fraction were not observed the presence of amide I vibration (1650 cm^{-1}) and amide II vibration (1550 cm^{-1}) characteristic of peptides. Based on IR spectra data obtained, which were compared with IR data for fatty acids reported by (Kiefer et al., 2010; Shapaval et al., 2014), the constituents presents in mucus from *P. boraceiensis* were proposed as fatty acids. Fatty acids are simples lipids found in natural products. The presence of fatty acids was also reported in mollusks (Zhukova, 2014, 2007).

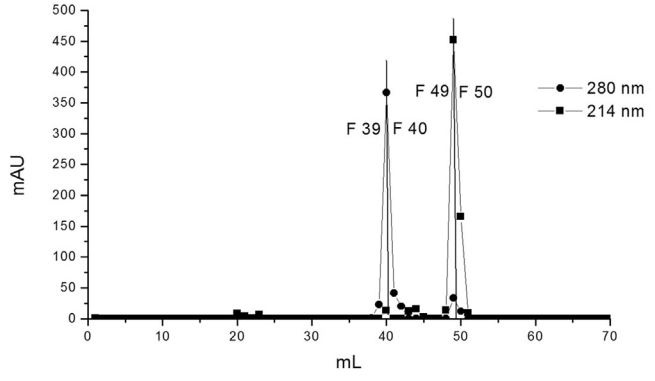

Fig. 1. Chromatographic profile of *Phyllocaulis boraceiensis* mucus obtained using an Akta Purifier high pressure chromatograph with a Superdex 75 gel filtration column. Four fractions (39, 40, 49 and 50) were selected for the MV antiviral test.

Fractions 39 and 50 were also analyzed by high performance liquid chromatography, which is powerful strategies to combine with mass spectrometry, in order to assign the structure of compounds. Most fatty acids show no useful absorption in the visible and ultraviolet (UV) region for detection by HPLC/DAD (Miwa, 2002; Tomer et al., 1983; Trufelli et al., 2011). The analysis of fatty acids were carried out primarily using electron ionization (EI); from saturated, straight chain species to more complex unsaturated and alkyl-substituted examples, which led to substantial ion decomposition followed by subsequent many steps of fragmentation. Recently, the technique of Electrospray ionization (ESI) is also used for analyses of polyunsaturated fatty acids (Murphy, 2014).

Electrospray ionization (ESI) of fatty acids produces molecular ion species in both positive $[M+H]^+$ and $[M + cation]^+$ as well as negative ions $[M - H]^-$. However, on the contrary of EI ionization led to little, if any, subsequent fragmentation. In this case, the fragmentation of molecular ion may be accomplished by collisional activation (collision induced dissociation, CID) with a neutral gas molecule in a collision cell (Murphy, 2014). Metal ion attachment to the carboxylate moiety $[M + H + Na]^+$ of fatty acids was used to generate positive ions from polyunsaturated fatty acids.

The alkali metal adducts $[M + H + Na]^+$ have a complete closed shell of electrons and are thus quite stable. In ion trap-type mass spectrometer, the excitation of this stable cation takes place during collisions with a neutral gas molecule, which is often relaxed by charge remote fragmentation mechanisms (Murphy, 2014). In reversed phase liquid chromatography, the elution order of fatty acid derivatives is mainly affected by the number of carbon atoms and the number of unsaturated bonds in the fatty acid chain (Bollinger et al., 2013; Hsu and Turk, 2008). Many different types of hydroxy fatty acids are formed as intermediates of fatty acid biosynthesis and metabolism. Monohydroxy fatty acids offered an additional channel for ion decomposition after collisional activation of positive ions, due to the lower activation energy imparted by the additional oxygen heteroatom (Murphy, 2014).

Table 1 and Fig. 2A summarizes the following information on the peaks observed during RPHPLC-ESI-MS/MS analyses: retention times (Rt), MS spectral data for sodiated and protonated molecules and proposed structures for polyunsaturated fatty acids **1**–**5**. The sodium adduct ions, $[M + H + Na]^+$ possess 22 mass atomic units above quasi-molecular ion in first-order mass spectra obtained with ESI in positive ion mode (Trufelli et al., 2011; Wilson et al., 2015; Xie et al., 2012).

In fraction 39 and 50, compounds **1**–**3** represent a homologous series of compounds, in which the molecular mass (MM) differed by 28 mass units. Compound **5** found only in fraction 50 possess 112 mass units (8 CH_2) more than compound **3**. The fragmentation pattern of compounds **1**–**3** is very similar and the product ions obtained following collisional activation of sodiated adduct $(M + H + Na)^+$ produce abundant fragments at m/z 270, attributed to the product ion $(NaC_{15}O_3H_{20})^+$ corresponding to the loss of 225 mass units and at m/z 298, which possess 28 mass units more than fragment ion at m/z 270. This fragmentation pattern also can indicate that compounds **1**–**5** are polyunsaturated fatty acids. For polyunsaturated fatty acid, a 1[5]-sigmatropic shift probably precedes the carbon-carbon cleavage step which alters the position of the closest double bond to an allylic position, that is more favorable to cleave. These allylic and/or vinylic carbon bond cleavages obtained with hydrogen transfer and formation of conjugated or neutral product ions are generated after collisional activation (Murphy, 2014).

The ESI-MS spectrum of compound **1**, exhibited sodiated molecule at *m/z* 495 (Table 1), indicating molecular mass (MM) corresponding to 472 g/mol, and was assigned as hydroxyhentriacontapentaenoic acid. Fatty acids possess many different

Table 1
RPHPLC-DAD-ESI-MS/MS analyses of mucus fraction 39 and 50: retention times (Rt), MS data for protonated molecules and proposed structures.

	Retention time	MS data protonated molecules	Proposed structures
1	48.7	$[M+Na]^+$ – 495 MM – 472 – $C_{31}O_3H_{52}$ MS/MS – 270 (100), 298 (20), 242 (20)	hydroxy-hentriacontapentaenoic acid.
2	49.4	$[M+Na]^+$ 523 MM – 500 – $C_{33}O_3H_{56}$ MS/MS – 270 (100), 298 (70)	hydroxy-tritriacontapentaenoic acid.
3	50.0	$[M+Na]^+$ 551 MM – 528 – $C_{35}O_3H_{60}$ MS/MS-298 (100)	hydroxy-pentatriacontapentaenoic acid.
4	53.3	$[M+Na]^+$ 413 MM – 390 – $C_{25}O_3H_{42}$ MS/MS-301 (100)	hydroxy-pentacosatetraenoic acid
5	53.6	$[M+Na]^+$ 685 $[M+H]^+$ 663 MM – 662 – $C_{45}O_3H_{74}$ MS/MS – 551 (100), 607 (80), 495 (70)	Oxo-pentatetracontaheptenoic acid

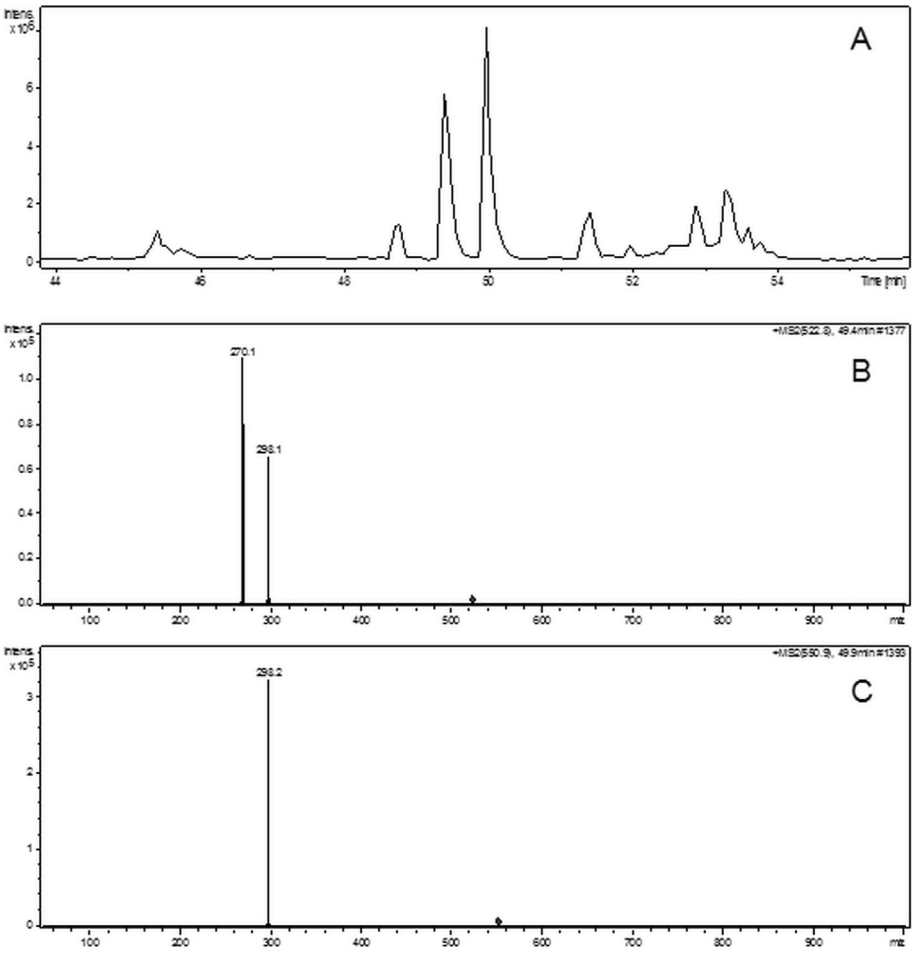

Fig. 2. Total ion current chromatogram obtained through ESI-MS analysis in positive ion mode from mucus extract of *P. boraceiensis* (A); ESI-MS/MS in positive íon mode – Compound **2** – hydroxy-tritriacontapentaenoic acid (B); ESI-MS/MS in positive íon mode – Compound **3** – hydroxy-pentatriacontapentaenoic acid (C).

complex species. Some polyunsaturated fatty acids exhibit similar molecular structures and consequently, their classification and discrimination is a challenging task from the analytical point of view. The determination of the position of the double bond is not possible using only mass spectral fragmentations data (Miwa, 2002; Thomas et al., 2014; Tomer et al., 1983; Trufelli et al., 2011). Generally, for polyunsaturated fatty acid derivatives, all double bonds have the cis (Z) configuration. The ESI-MS spectrum of compound **2** (Table 1, Fig. 2B) exhibited a sodiated molecule at *m/z* 523, corresponding to MM of 500 g/mol. Compound **2** was assigned as hydroxy-tritriacontapentaenoic acid. For compound **3** (Table 1, Fig. 2C), the ESI-MS spectrum showed a sodiated molecule at m/z 551, corresponding to an MM of 528 g/mol, and was assigned as hydroxy-pentatriacontapentaenoic acid. For compound **4**, the ESI-

MS spectrum exhibited sodiated molecule at m/z 413, which correspond to MM of 390 g/mol. After MS/MS experiments, the precursor ion at m/z 413 produced abundant fragment ion at m/z 301, attributed to the loss of 112 mass units (8 CH_2). Compound **4** was assigned as hydroxy-pentacosatetraenoic acid.

Compounds **2** and **3** were the main constituents present in fraction 39, while compounds **1** and **4** were found at low contents. In fraction 50, compounds **1**–**4** were found in low contents. Compound **5** is the main constituent present in fraction 50, which probably is responsible for the strong absorption band at 1731 cm^{-1} in the infrared spectrum (FTIR), attributed to (C═O) stretching. Compound **5** exhibited sodiated molecule at m/z 685 and protonated molecule at m/z 663, corresponding to MM of 662 g/mol. After MS/MS experiments the precursor ion at *m/z* 663 showed product ions at m/z 607 (80), m/z 551 (100) and m/z 495 (70); in which each fragment ions correspond to the loss of 56 mass units (4 CH_2). In this case, from the highest mass-to-charge ratio formed by the fragmentation mechanism initiated at a different allylic/vinylic site for the hydrogen transfer, the other n-series ions are observed at sequential loss of 56 mass units intervals. Compound **5** was assigned as oxo-pentatetracontaheptaenoic acid. This approach allowed only a tentative identification for compounds **1**–**5**. The full structural information must be confirmed through NMR ^{1}H and ^{13}C analysis.

Determination of the cytotoxicity of *Phyllocaulis boraceiensis* mucus by the MTT colorimetric method.

The cytotoxicity of *P. boraceiensis* mucus was determined by the MTT colorimetric method. The results show that *P. boraceiensis* mucus was not significantly toxic to Vero cells (Fig. 3) and the lethal concentration (IC50%) was 41, 73.2 and 92.6 μL when cells were treated with the crude mucus, fraction 39 and fraction 50, respectively.

3.2. Antiviral activity against measles virus

In order to determine the antiviral effect of *P. boraceiensis* mucus, Vero cells were treated with 2% total mucus and its fractions (39, 40, 49 and 50) and infected with MV. As can be seen in Fig. 4, the total *P. boraceiensis* mucus, as well as fractions 39 and 50, were able to reduce the infection of MV. In the image, the presence of syncytia can be observed; this is characteristic of the cytopathic effect of MV and the cells appear infected. The others fractions did not inhibit the replication of MV. In addition, Vero cells infected and treated with mucus and fractions 39, 40, 49 and 50were processed by qPCR. The results show that fractions 39 and 50 significantly reduced the number of copies of MV genomic DNA in the cell lysate (Fig. 5). Both fractions 39 and 50 inhibited the replication of MV, but fraction 50 exhibited lower MV antiviral activity, when compared to fraction 39. These results suggest that the *P. boraceiensis* mucus may have additional inhibitory effects on viral mRNA synthesis.

3.3. Determination of antiviral activity against infected cell cultures using fluorescent antibodies

Fig. 6 shows the antiviral effect of *P. boraceiensis* mucus and fractions 39, 40, 49 and 50 in cultures infected with MV. Our results showed that cells infected and treated with fraction 39, demonstrated significant reduction in viral infection of MV. The other fractions showed no significant reduction in viral infection. Slides were analyzed by Axio microscope (200× magnification).

3.4. Direct electron microscopy (DEM)

Electron micrographs of MV treated with fractions 39 and 50 of *P. boraceiensis* fractions are shown in Fig. 7. MV before (Fig. 7A and B) and after treatment with fractions 39 and 50 (7C, 7D) were submitted to identical treatment. Fig. 7A and B shows the ultrastructural appearance of a single virus particle, or virion, of MV. In Fig. 7C and D, MV was observed using an electron microscope, with evident disruption of the lipoprotein envelope of MV following treatment with fractions 39 and 50, which contain the polyunsaturated fatty acids **1**–**5** as the main constituents. Some studies have demonstrated the antiviral action of unsaturated fatty acids on enveloped viruses, which disrupt both viral envelopes and cell membranes. It is known that polyunsaturated fatty acids affect only the viral envelope of viruses, causing leakage, which is increased using higher concentrations of these compounds; this can lead to complete disintegration of the envelope and the viral particle (Kohn et al., 1980; Thormar et al., 1987). Thus, the loss of infectivity was attributed to disruption of the lipoprotein envelope of MV.

4. Discussion

The search for antiviral compounds has involved carrying out assessment of the exudates of many invertebrate organisms. *Phyllocaulis boraceiensis* mucus is an exudate that acts as the animal's defense system. This material is a glycoprotein that “in natura” appears highly viscous. Thus, these experiments were carried out using organic solvents (Toledo-Piza et al., 2013).

Recent studies have demonstrated the importance of research that investigates the antiviral activity of exudates extracted from invertebrates, mainly with emphasis on mollusks in the search for new antiviral compounds. Our research group identified several proteins in the hemolymph of *Lonomia oblique* with antiviral (Carmo et al., 2012; Greco et al., 2009) and antiapoptotic (Souza et al., 2005) activity, as well as enzymes responsible for increasing cell longevity (Maranga et al., 2003). Also, the hemocyanin isolated from the marine mollusk *Haliotis rubra* exhibited antiviral activity against herpes simplex virus type 1 (Zanjani et al., 2014). A polysaccharide isolated from the shell fish *Cipangopaludina chinensis* exhibited antiviral action against hepatitis B virus (X.-Y. Liu et al., 2013b). The egg wax of *Amblyomma cajennense* ticks also exhibited antiviral activity (De Lima-Netto et al., 2012). The peptide miticin class C, isolated from the mollusk *Mytilus galloprovincialis*, exhibited antiviral action in cultures of viral hemorrhagic septicemia virus (Balseiro et al., 2011). The hemolymph isolated from the caterpillar *Lonomia oblique* showed antiviral activity in cultures containing measles, influenza and poliovirus (Greco et al., 2009). The antiviral action of glycopeptides from the mollusk *Rapana venosa* has observed in cultures of respiratory syncytial virus (Dolashka-Angelova et al., 2009). The polyethers aplysqualenol A and B, extracted from the marine mollusk *Aplysia dactylomela*, exhibited antiviral activity against herpes virus cultures (Vera et al., 2009).

Measles virus, a member of the genus *Moribilli virus* from the *Paramyxoviridae* family, is a highly pathogenic non-segmented negative-stranded RNA virus that causes respiratory distress and immune suppression after infection of the human host. The MV genome encodes a membrane-associated matrix (M) protein, the hemagglutinin (H) and fusion (F) envelope glycoproteins, the RNA polymerase associated phosphoprotein (P) and large polymerase (L) protein, and the nucleocapsid (N) protein that surrounds the viral genome. Three known entry receptors have been identified to date for both laboratory-adapted strains and clinical isolates of MV: CD46 (membrane cofactor protein, MCP), CD150/signaling lymphocyte-activation molecule (SLAM) and, most recently, poliovirus receptor-like protein 4 (PVRL4), also known as nectin (Dörig et al., 1993; Hsu et al., 2001).

Despite its large and longstanding impact on global public health, there is currently no effective antiviral treatment available

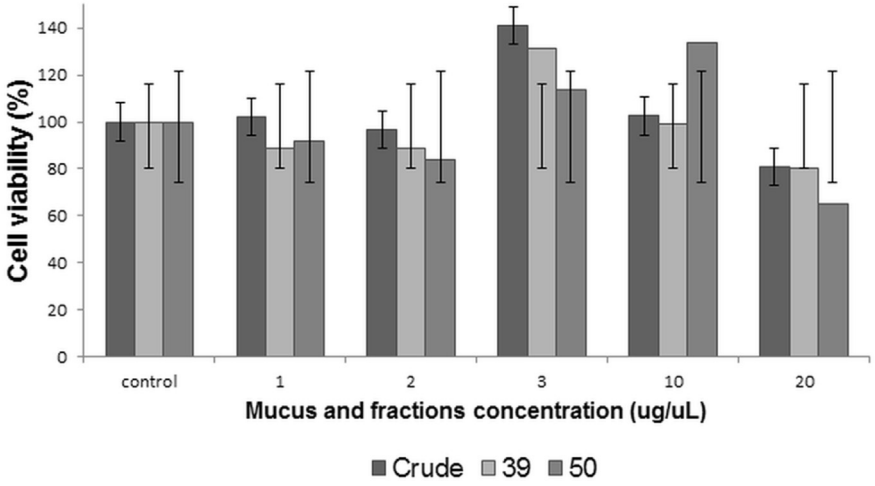

Fig. 3. Viability/cellular proliferation evaluation using the MTT test in Vero cells treated with the crude mucus of *Phyllocaulis boraceiensis* and fractions 39 and 50. The error bars represent the standard deviation (SD) from three replicates for each set of values.

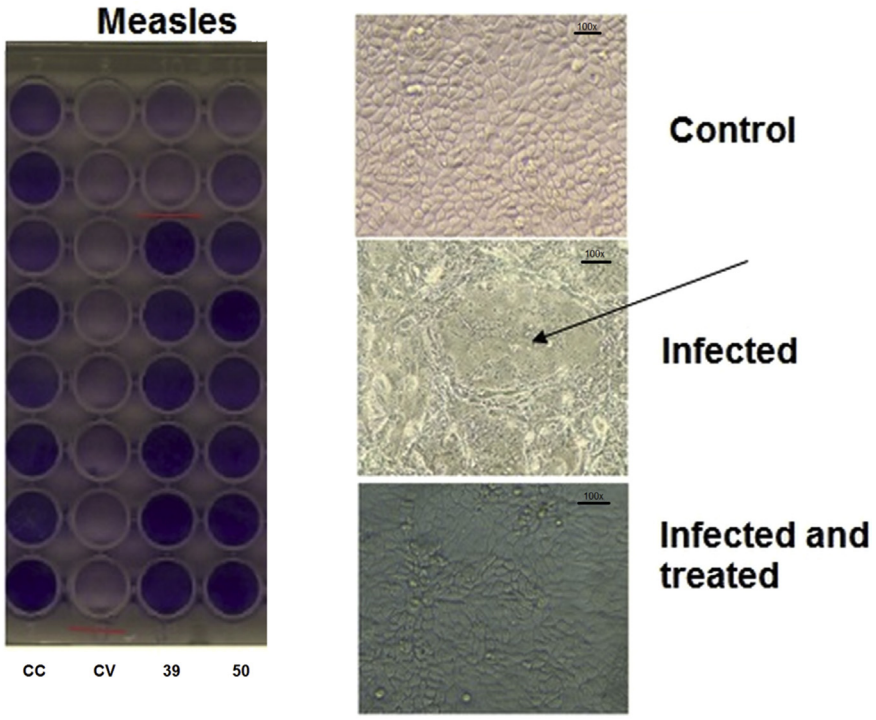

Fig. 4. Determination of the antiviral activity of *P. boraceiensis* mucus on Vero cells infected with measles virus (Edmonston strain). Cells were treated with or without 2% mucus and infected with MV. The plate was assessed daily by observing the appearance of a cytopathic effect. After 5 days, the cultures were stained with crystal violet. Control cells (CC), virus control (CV), fraction 39 and fraction 50. A syncytium formed by the virus is indicated by an arrow. Results are representative of experiments performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for MV. A therapeutic approach for treating severe measles infections could involve the discovery of new natural products, particularly for immune compromised individuals. The outcome of a viral infection results from a combinatorial interaction network of multiple viral, cellular and host factors. One of them is the interferon response, which limits virus spread and initiates the adaptive immune response (Fontana et al., 2008). In the present study, we tested viral inhibition by *Phyllocaulis boraceiensis* mucus and its fractions by assessing the replication and growth of wild-type MV. Using an antiviral assay, our results show a 64-fold reduction in the growth of MV when treated with fraction 39; using a qPCR assay, this reduction was almost 80%. The inhibition of MV was also observed in electron micrographs.

As far as we know, there is not information about the chemical composition of mucus from *P. boraceiensis*. The results obtained using IR and mass spectra data indicated the presence of polyunsaturated fatty acids (PFA) as the main constituents in fractions 39 and 50 from *P. boraceiensis* mucus. The FTIR spectra were used in a study carried out by (Kiefer et al., 2010) to discriminate two fatty acids, eicosapentaenoic acid and arachidonic acid, which show strong similarities in chemical structure. Polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid, are

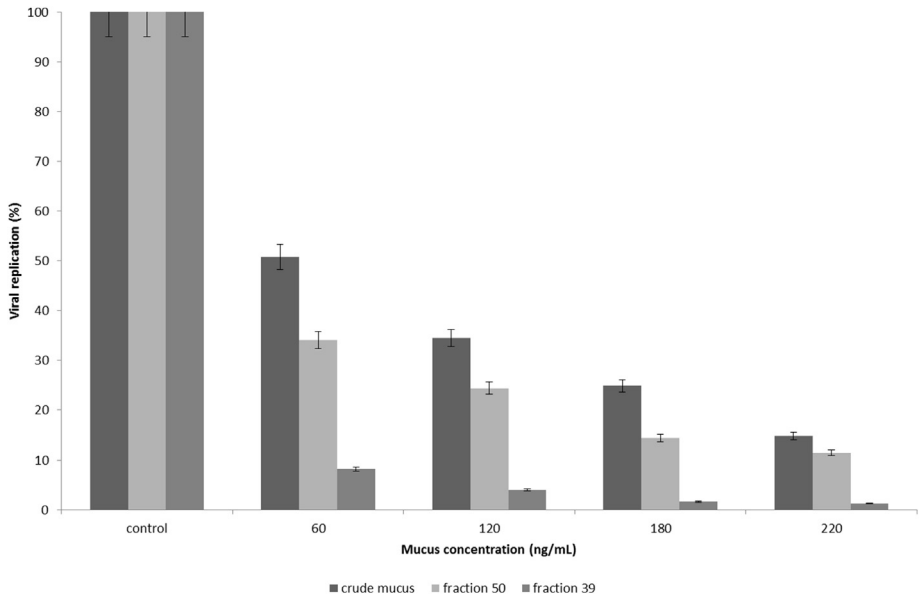

Fig. 5. Inhibitory effect of *P. boraceiensis* mucus, fraction 39 and 50 on MV virus RNA synthesis in Vero cells analyzed by qRT-PCR. Cell culture lysates were collected after pre-treatment with different concentrations from *P. boraceiensis* mucus for 72 h. The inhibitory effect was determined using qPCR. The infectivity from MV decreases after pre-treatment with *P. boraceiensis* mucus. The error bar represents the SD calculated from three independent experiments in triplicate. Note: Pre-treatment inhibitory infection was more 90%.

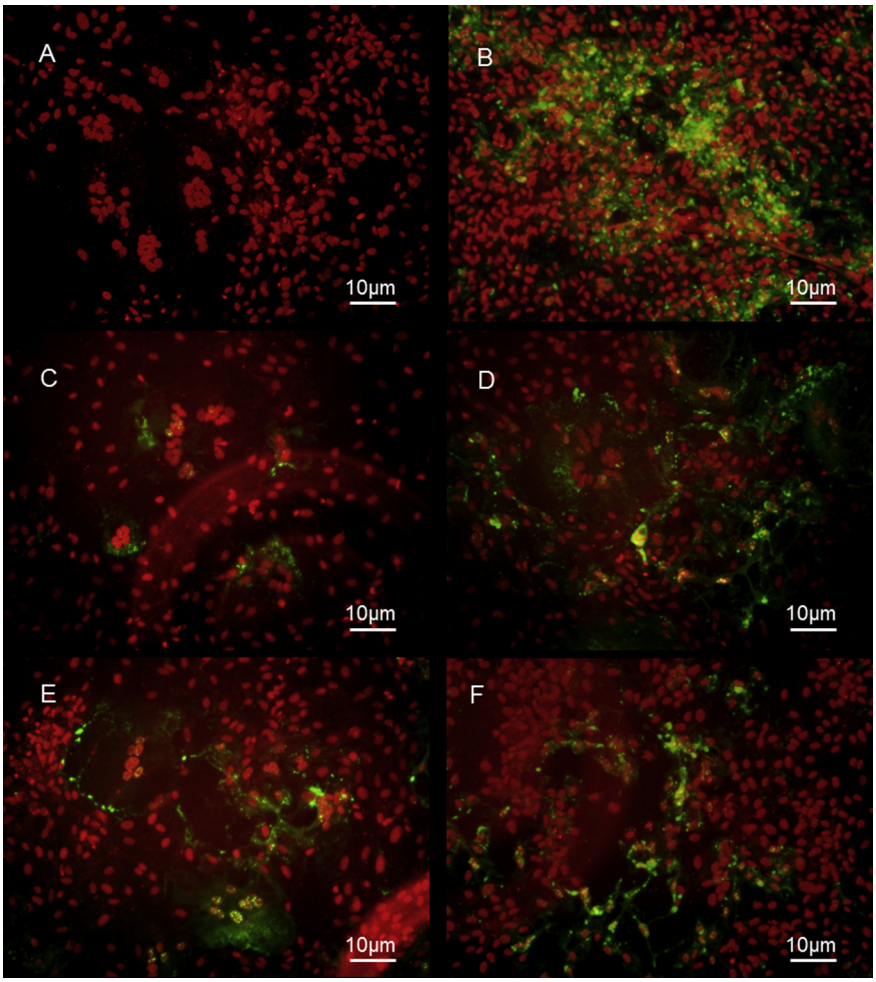

Fig. 6. Fluorescence microscopy of Vero cells infected with measles virus and treated with purified fractions of *P. boraceiensis* mucus. The cells were labeled with anti-measles antibody (green) and propidium iodide (red). Uninfected cells (A); cells infected but not treated (B); cells infected and treated with fractions 39 (C), 40 (D), 49 (E) and 50 (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

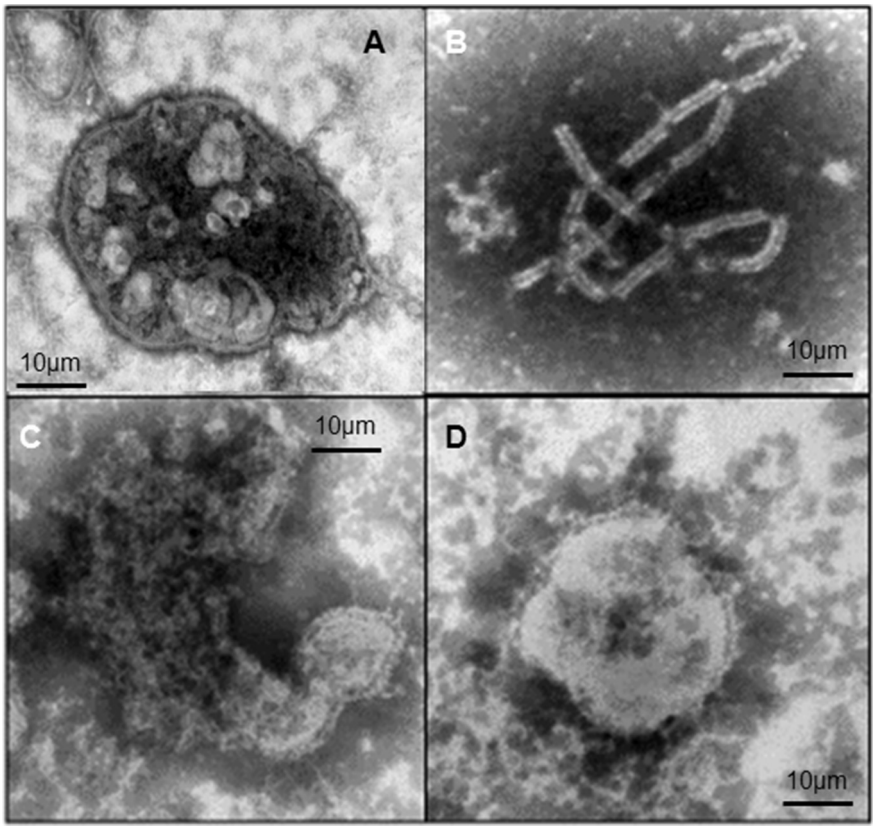

Fig. 7. Electron micrographs of measles virus (MV) treated with fractions 39 and 50. Image A and B show the ultrastructural appearance of a single virus particle or virion of MV. Image C and D show MV treated with fractions 39 and 50. *Note: Disintegration of the MV cell membrane.

generally converted into oxygenated derivatives known as eicosanoids and docosanoids which exhibited potent biological activity (Bollinger et al., 2013; Hsu and Turk, 2008).

Fatty acids, especially unsaturated fatty acids, are important as nutritional substances and metabolites in living organisms, and are widely distributed in food, living organisms and biological fluids (Bollinger et al., 2013; Liu et al., 2013a,b; Trufelli et al., 2011; Xing et al., 2014). Many kinds of fatty acids play important roles in the regulation of a variety of physiological and biological functions. Saturated fatty acids are involved in energy production, energy storage, lipid transport, the synthesis of phospholipids and sphingolipids and the modification of many regulatory proteins (Tomer et al., 1983; Trufelli et al., 2011). Besides this, long chain unsaturated fatty acids with two or more double bonds carry out important roles in reducing the risks of cancer, heart disease, cardiovascular disease, autoimmune and inflammatory disorders and disrupted neurological functions (Liu et al., 2013a,b; Xing et al., 2014). Several polyunsaturated fatty acids including arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) exhibited anti-HCV activities using an HCV sub genomic RNA replicon system (Leu et al., 2004).

MV can inhibit interferon synthesis within an infected cell, but it can also block its release and prevent the establishment of an antiviral state in neighboring uninfected cells. Exposure of enveloped viruses such arbo-, myxo-,paramyxo- or herpesviruses to micromolar concentrations of fatty acids cause a rapid loss of infectivity of viruses (Kohn et al., 1980). Fatty acids that are normal components of lipids, when tested against enveloped viruses, i.e. vesicular stomatitis virus, herpes simplex virus and visnavirus, affected the viral envelope, causing leakage; what was increased at higher concentrations, leading to the complete disintegration of the envelope and the viral particles (Thormar et al., 1987). We speculate that the antiviral activity of *Phyllocaulis boraceiensis* mucus could be attributed to the action of PFA on the viral envelope, because the loss of infectivity was attributed to a disruption of the lipoprotein envelope of these virions, as observed by direct electron microscopy (See Fig. 6). In these experiments, we demonstrated a direct effect of PFA on MV. However, the mechanism of action, likely due to the interaction between PFA and the envelope of the virus, is still obscure. Beside this, PFA can change the plasma membrane of cultured cell, thus preventing virus entry.

5. Conclusion

Phyllocaulis boraceiensis mucus contains polyunsaturated fatty acids in fraction 39, which exhibited antiviral activity against MV, as shown by qPCR and direct electron microscopy. The main constituents found in fraction 39, the most active fraction against MV were assigned as hydroxy-tritriacontapentaenoic acid (**2**) and hydroxy-pentatriacontapentaenoic acid (**3**). Fraction 50 exhibited lower antiviral activity against MV. In fraction 50, compounds 2 and 3 were also found, although at lower contents. The main constituent present in fraction 50 was oxo-pentatetracontaheptenoic acid (**5**). Some studies demonstrated that the antiviral action of PFA on enveloped viruses occurs due to the fact that these compounds disrupt both viral envelopes and cell membranes. The antiviral effect observed on MV was attributed to disintegration of the viral envelope by PFA, which are incorporated into the lipid membrane and destabilize the bilayer.

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