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Anti-Mayaro virus activity of *Cassia australis* extracts (Fabaceae, Leguminosae)

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Abstract

Background: The arthropod-borne Mayaro virus (MAYV) causes 'Mayaro fever', a disease of medical significance, primarily affecting individuals in permanent contact with forested areas in tropical South America. Studies showed that the virus could also be transmitted by the mosquito *Aedes aegypti*. Recently, MAYV has attracted attention due to its likely urbanization. To date, there are no drugs that can treat this illness.

Methods: Fractions and compounds were obtained by chromatography from leaf extracts of *C. australis* and chemically identified as flavonoids and condensed tannins using spectroscopic and spectrometric techniques (UV, NMR, and ESI-FT-ICR MS). Cytotoxicity of EtOAc, *n*-BuOH and EtOAc-Pp fractions were measured by the dye-uptake assay while their antiviral activity was evaluated by a virus yield inhibition assay. Larvicidal activity was measured by the procedures recommended by the WHO expert committee for determining acute toxicity.

Results: The following group of substances was identified from EtOAc, *n*-BuOH and EtOAc-Pp fractions: flavones, flavonols, and their glycosides and condensed tannins. EtOAc and *n*-BuOH fractions inhibited MAYV production, respectively, by more than 70% and 85% at 25 µg/mL. EtOAc-Pp fraction inhibited MAYV production by more than 90% at 10 µg/mL, displaying a stronger antiviral effect than the licensed antiviral ribavirin. This fraction had an excellent antiviral effect (IC₉₀ = 4.7 ± 0.3 µg/mL), while EtOAc and *n*-BuOH fractions were less active (IC₉₀ = 89.1 ± 4.4 µg/mL and IC₉₀ = 40.9 ± 5.7 µg/mL, respectively).

Conclusions: *C. australis* can be used as a source of compounds with anti-Mayaro virus activity. This is the first report on the biological activity of *C. australis*.

Keywords: *Cassia australis*, Flavonoids, Tannins, Antiviral, MAYV, Larvicidal activity, *Aedes aegypti*

Background

In Brazil, MAYV is endemic in the Amazon region, but Mayaro fever outbreaks have occurred in other regions in Brazil [1,2]. Most arboviruses isolated in the Amazon region are maintained in nature by different life cycles, involving different vectors and vertebrate hosts. Oro-pouche virus, for example, is transmitted to humans in urban areas by the midges *Culicoides paraensis*, and vertebrates such as sloths, monkeys and birds play a role in the maintenance of the virus cycle [3]. Likewise, Mayaro and yellow fever viruses are transmitted by the mosquito *Haemagogus janthinomys* in the jungle, and monkeys are

the main hosts [4]. On the other hand, dengue virus has a simpler cycle whereby the serotypes are directly transmitted to humans by *Aedes aegypti* mosquito bites [5]. In addition, imported MAYV cases in other countries from tourists who visited the Amazon region have been described [6]. MAYV can also be transmitted by the vectors *Aedes aegypti* and *A. albopictus*, which raises the concern for urban areas. It is very important to point out that MAYV is closely related to the Chikungunya virus, also transmitted by the mosquitoes *Aedes aegypti* and *A. albopictus*, which has recently reached Europe and the Americas and is now counting nearly 800 of autochthonous infections in Brazil since the first detected case in this country in August 2014 [7,8].

Natural products are becoming very attractive because of their low cytotoxicity, the rapid degradation in the

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environment, and because of the complexity of the chemistry in these products, that should limit resistance and increase the applicability of use, such as vector control studies [9].

The majority of the available antiviral drugs are concentrated in a small number of viruses, such as HIV, Herpes and Influenza [10]. Nevertheless, research efforts to explore the potential of natural products as sources of novel low toxicity and high selectivity antiviral substances have increased lately [11]. Because there are many approaches for the use of natural products, the modes of action or the active components they contain and the metabolic pathways they interact must be studied. This can be accomplished initially by *in vitro* studies such as the cell culture approach in this paper.

The genus *Cassia* (Fabaceae, Leguminosae) comprises more than 600 species including shrubs, trees and herbs, distributed in tropical and subtropical regions all over the world. The separation of the genus *Senna* from the genus *Cassia* has been, and still is, the subject of many discussions [12]. The species under study was originally classified as *Cassia australis* Vellozo and in 1982 by Irwin and Barneby it was transferred to *Senna* with the name: *Senna australis* (Vell.) H.S.Irwin & Barneby. Afterwards, this species has been renamed *Senna appendiculata* (Vogel) [13].

In Brazilian ecosystems, particularly in the Atlantic forest, the genus *Senna* is widespread, with some species in the Southeast greatly appreciated for the beauty of its flowers, and therefore widely used as ornamental plants [14]. Due to the traditional use, several species, many already described in the literature, are medicinally used worldwide [15-20]. *Cassia australis* is a medium sized shrub and may reach up to 2.5 meters of diameter. It occurs at Brazilian coast sandbank, mainly in Rio de Janeiro, Espirito Santo, Bahia, Sergipe, Alagoas and Pernambuco states [21]. The genus *Cassia* is known for the presence of a variety of compounds. Anthraquinones are the main class of compounds isolated from it [17,22-25]. However, previous investigations led to the isolation of flavonoids [26-29], piperidine alkaloids [30], stilbenoids [30] and aliphatic esters [16]. So far, there is no paper about the phytochemistry and biological activity of *Cassia australis*.

For most mosquito-transmitted viruses, there are no licensed antiviral drugs or vaccines available. MAYV is an example of an arthropod-borne virus, mainly found in South America tropical areas, which affects primarily individuals in permanent contact with forested areas and causes the Mayaro fever. In this study, EtOAc, *n*-BuOH and EtOAc-Pp fractions containing flavonoids and other classes of phenolics compounds were obtained from the leaves of *Cassia australis* Vellozo and investigated for their *in vitro* antiviral activity against MAYV replication

in Vero cells and larvicidal activity against *Aedes aegypti* larvae.

Methods

Plants, cells and viruses

Cassia australis leaves were collected in December 2008 in Rio de Janeiro State, and identified by Alice Sato. Voucher specimens (No. 652HUNI) are deposited in the herbarium of the University of Rio de Janeiro (UNIRIO), Brazil.

Vero cells (African green monkey kidney, ATCC CCL-81) were maintained at 37°C, 5% CO₂, in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, USA) supplemented with 5% fetal bovine serum (Cultilab, BRA), 50 IU/mL of penicillin, and 50 µg/mL of streptomycin (Sigma-Aldrich, USA). Mayaro virus (ATCC VR-66, lineage TR 4675) was propagated in Vero cells and viral stocks kept at -70°C until use.

Aedes aegypti eggs were obtained at the Brazilian Army Institute of Biology. They were kept in the tray containing tap water at optimal conditions (28 ± 1°C). After 48 hrs of incubation, the eggs were used. The 4th instar larvae were used in the study.

Extraction, fractionation, and purification for achievement of fractions and compounds

Air-dried leaves (850 g) were extracted with MeOH:H₂O (8:2) at room temperature by static maceration over 10 days. After concentration under reduced pressure, the methanol extract (25 g) was suspended in MeOH:H₂O (9:1), and partitioned with hexane. After removal of the methanol from the defatted extract, the remaining aqueous solution was partitioned successively with CH₂Cl₂, EtOAc, and *n*-BuOH. Two grams of the EtOAc extract, soluble in H₂O:MeOH (9:1), were applied to a XAD-2 column (procedure A), and chromatographed in a step-wise gradient with H₂O:MeOH (10:0/0:10). 150 ml of each combination of solvents were eluted through the column and fractions of 150 ml were collected. The fraction obtained with 100% of water was named EtOAc-Pp. From H₂O:MeOH (50:50) fraction, after chromatography on Sephadex LH-20 (MeOH:H₂O - 1:1, mobile phase) - (procedure B), the flavone tricetin-4'-methoxy-3'-β-glucoside [31] was obtained. The same procedures A and B were applied to *n*-BuOH extract to obtain the flavone vicenin-2 [32].

Reverse-phase HPLC-DAD-UV, TLC, NMR and ESI(-)-FT-ICR MS analyses

HPLC-DAD-UV (High Performance Liquid Chromatography with Diode Array Detector), TLC (Thin Layer Chromatography), NMR (Nuclear Magnetic Resonance) and ESI(-)-FT-ICR MS (Electrospray ionization with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry)

were used to analyze the chemical composition of EtOAc, *n*-BuOH, EtOAc-Pp fractions and compounds isolated from them.

The mobile phase for HPLC-DAD analysis consisted of solvent (A) 1% phosphoric acid in water and solvent (B) 1% phosphoric acid in methanol and was used under the following gradient: 5% of B to 70% of B in A for 55 min. The flow rate was 1 mL/min and the injection volume 20 μ L. The UV-vis spectra were recorded from 210 to 400 nm and the detector focused on 254 and 365 nm. TLC was performed on silica gel plates 60 F₂₅₄ (Merck, 20×20 cm, 0.5 mm thickness), using *n*-butanol-water-acetic acid (4:5:1) and chloroform-methanol (9:1) as mobile phases. After elution, TLC plates were observed under 254 nm UV light and then sprayed successively with solutions of NP (2-aminoethyl-diphenylborinate 1% in methanol) and PEG-4000 (polyethylene glycol 5% in ethanol) (both by Sigma-Aldrich, USA) before examination under 365 nm UV light. *Cassia australis* extracts and pure compounds were analyzed by an ultra-high resolution and accuracy mass spectrometer (model 9.4 T Solarix, Bruker Daltonics, Bremen, Germany). Briefly, the samples were dissolved in acetonitrile/ammonium hydroxide (99.9/0.1 v/v %) mixture to a final concentration of 10 μ g mL⁻¹. The mass spectrometer was set to operate in negative ion mode, ESI(-), over a mass range of *m/z* 200–2000. The parameters of the ESI(-) source were as follows: nebulizer gas pressure of 0.5–1.0 bar, capillary voltage of 3–3.5 kV, and transfer capillary temperature of 250°C. The spectrum was processed using the Compass Data Analysis software package (Bruker Daltonics, Bremen, Germany). A resolving power, $m/\Delta m_{50\%} \approx 500\,000$, in which $\Delta m_{50\%}$ is the full peak width at half-maximum peak height, of *m/z* ≈ 400 and a mass accuracy of <1 ppm provided the unambiguous molecular formula assignments for singly charged molecular ions. Elemental compositions of the compounds were determined by measuring the *m/z* values. NMR analysis (¹H-NMR, COSY, HSQC and HMBC) were recorded on Varian spectrometer MR-400 operating at 400 MHz. The samples were solubilized in DMSO-d₆ and TMS was used as external standard.

Final compound analysis was performed by NMR (DMSO-d₆), FT-ICR-ESI-MS, UV spectral analysis, and by comparison with literature values.

Cytotoxicity assay

Cytotoxicity analysis was performed using the dye-uptake method modified from Borenfreund and Puerner [33]. Vero cells grown in 96-well microplates were treated with culture media containing different concentrations of the substances. After 24 hours, the medium was replaced by a solution of 50 μ g/mL neutral red, the cells were incubated for 3 hours at 37°C, 5% CO₂ and then fixed and extracted with 20% formaldehyde and 50% methanol, 1%

acetic acid. Absorbance was read at 490 nm, using a spectrophotometer, to detect neutral red incorporation by living cells. Absorbance results were used to calculate, by regression analysis, the concentrations of the tested substances capable of reducing cell viability by 50% and 90% relative to controls (CC₅₀ and CC₉₀, respectively).

Antiviral activity assay

For the antiviral activity, confluent Vero cell monolayers grown in 24-well plates were infected with MAYV (multiplicity of infection = 0.1) for 1 hour, then rinsed with PBS and treated for 24 hours (at 37°C and 5% CO₂) with different concentrations (0–100 μ g/ml) of the substances diluted in culture medium. After treatment, culture supernatants were recovered and used for titration of extracellular infectious virus particles. Ribavirin (Sigma-Aldrich, USA) was used as positive control for MAYV replication inhibition. For each substance or extract, IC₅₀ and IC₉₀ values were calculated and used to obtain a selectivity index (SI), expressed as the ratio CC₅₀/IC₅₀, and to estimate relative potency (RP) as the ratio between ribavirin (reference substance) IC₉₀ and the tested substance's IC₉₀. Results were presented as mean inhibitory/cytotoxic concentration \pm SD, and *t*-tests were used to evaluate the statistical significance of treatments relative to controls. *P*-values <0.05 were considered statistically significant.

Virus yield assay

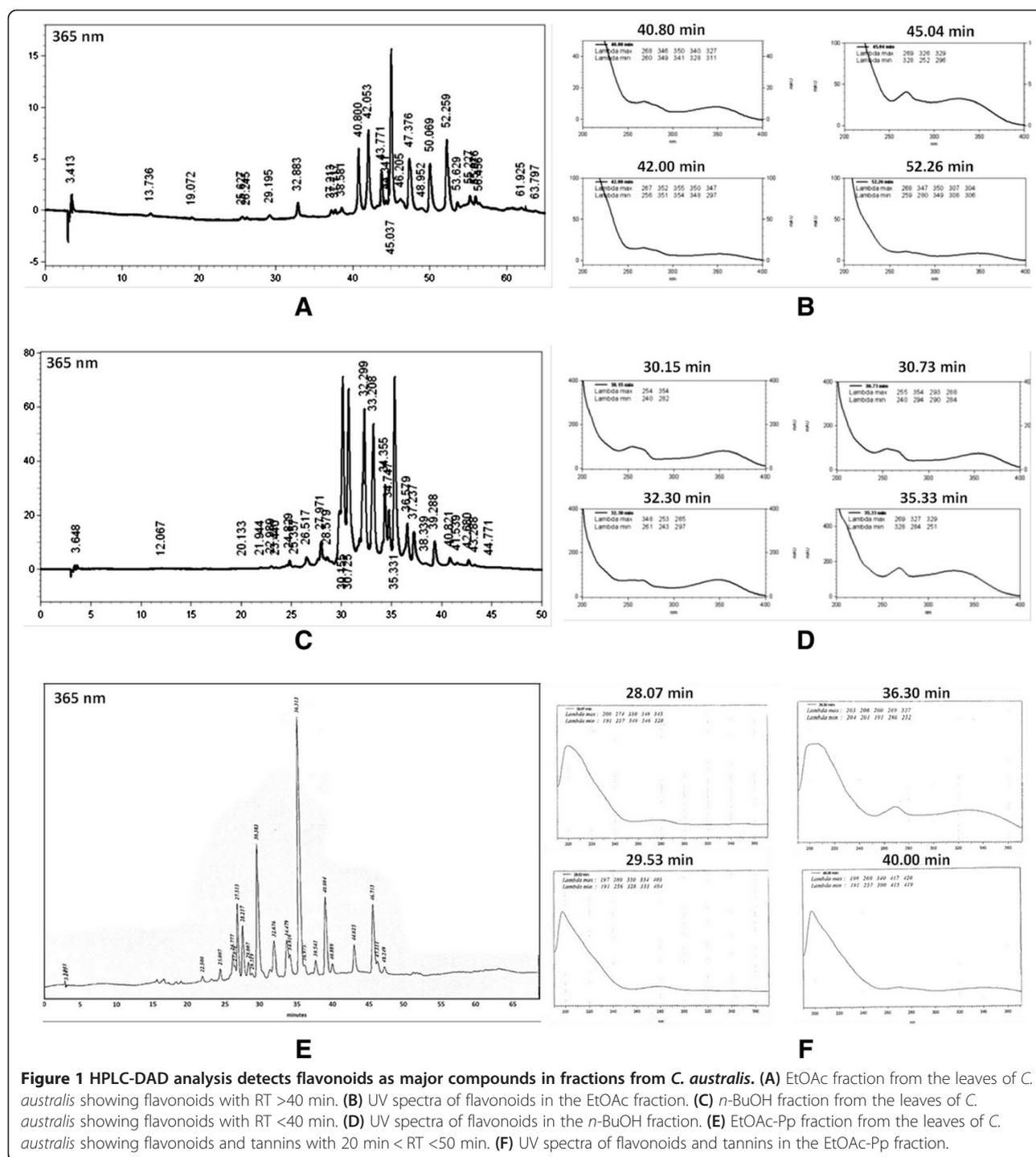
For virus titration, confluent cell monolayers in 24-well plates were infected with serial dilutions of recovered supernatants from the assays for 1 hour at 37°C, 5% CO₂. After inoculum removal, cells were rinsed with PBS and the monolayer was incubated with fresh medium with 2% carboxymethylcellulose (Sigma-Aldrich, USA) for 48 hours at 37°C, 5% CO₂. Finally, cells were fixed with 20% formaldehyde and stained with 0.5% crystal violet in 20% ethanol, and viral plaques were counted.

Results

Flavonoid aglycones, flavonoid glycosides and tannins were found in extracts of *C. australis* leaves

HPLC-DAD-UV analysis of EtOAc, *n*-BuOH and EtOAc-Pp fractions indicated different flavonoid profiles. Flavonoid aglycones and flavonoid monoglycosides with retention time (RT) greater than 40 min., predominated in EtOAc fraction (Figure 1A and B), while flavonoid diglycosides, more polar compounds (30 min. < RT <40 min) (Figure 1C and D), predominated in *n*-BuOH fraction. HPLC-DAD analysis of EtOAc-Pp showed more polar compounds (20 min < RT <50 min), like tannins and flavonoid diglycosides (Figure 1E and F).

All the ESI(-)FT-ICR MS analyses (Table 1) were made in negative ion mode. The structures were suggested based on their ultra-high resolution and accuracy mass.



Molecular formula (M) and double bond equivalent (DBE) were utilized to propose the presence of flavonols, flavones, and their glycosides and condensed tannins (dimer and trimer of flavan-3-ol). EtOAc fraction showed presence of flavonols (*m/z* 285, 301 and 315), condensed tannins (*m/z* 529, 545 and 561), flavonol glycosides (*m/z* 447 and 463) and flavone glycoside (*m/z* 477). All were detected in deprotonated form, $[M - H]^-$ ion. From the

n-BuOH fraction were proposed flavonol glycosides (*m/z* 447, 463, and 609) and flavone glycosides (*m/z* 477 and 593) while the EtOAc-Pp showed the presence of flavonols and flavanones (*m/z* 285, 289, 301 and 315), flavonol glycosides (*m/z* 447 and 463), flavone glycoside (*m/z* 477) and condensed tannins (*m/z* 513, 529, 545 and 769). The chemical structure of compounds identified is proposed in Table 1.

Table 1 Proposed compounds from ESI(-)FT-ICR MS analyzes

Suggested compounds	Class of natural product	EtOAc fraction	<i>n</i> -BuOH fraction	EtOAc-Pp	$m/z_{theoretical}$	$m/z_{measured}$	Molecular formula (M)	Error (ppm)	DBE
Kaempferol	Flavonol	+	-	+	285.04046	285.04042	C ₁₅ H ₁₀ O ₆	0.41	11
Quercetin	Flavonol	+	-	+	301.03538	301.03538	C ₁₅ H ₁₀ O ₇	0.25	11
Rhamnetin/isorhamnetin	Flavonol	+	-	+	315.05103	315.05098	C ₁₆ H ₁₂ O ₇	0.33	11
Quercetin pentoside	Flavonol Monoglycoside	+	+	+	447.09329	447.10361	C ₂₁ H ₂₀ O ₁₁	0.38	12
Quercetin hexoside	Flavonol Monoglycoside	+	+	+	463.08820	463.08801	C ₂₁ H ₂₀ O ₁₂	0.38	12
Tricetin-4'-methoxy-3'- β -D-glucoside ¹	Flavone Monoglycoside	+	+	+	477.10385	477.10361	C ₂₂ H ₂₂ O ₁₂	-0.12	12
-	Flavan-3-ol dimer	+	-	+	529.15041	529.15024	C ₃₀ H ₂₆ O ₉	0.51	18
-	Flavan-3-ol dimer	+	-	+	545.14532	545.14519	C ₃₀ H ₂₆ O ₁₀		18
-	Flavan-3-ol dimer	+	-	-	561.14024	561.08601	C ₃₀ H ₂₆ O ₁₁	0.45	18
-	Flavan-3-ol dimer	+	-	-	591.11441	591.09653	C ₃₀ H ₂₄ O ₁₃	0.40	19
Vicenin-2 kaempferol diglycoside ¹	Flavone diglycoside	-	+	+	593.15120	593.15104	C ₂₇ H ₃₀ O ₁₅	-0.08	13
Quercetin dihexoside	Flavonol diglycoside	-	+	+	609.14611	609.14591	C ₂₇ H ₃₀ O ₁₆	0.06	13
-	Flavan-3-ol trimer	-	-	+	769.22905	769.22868	C ₄₅ H ₃₈ O ₁₂	0.08	27
-	Flavan-3-ol trimer	-	-	+	785.22397	785.22349	C ₄₅ H ₃₈ O ₁₃	0.08	27

¹Purified compounds identified by NMR, DBE - double bond equivalent, + detected, - not detected, Mass error (ppm) = $[(m/z_{measured} - m/z_{theoretical})/m/z_{theoretical}] * 10^6$.

Cytotoxicity and antiviral activity

EtOAc, *n*-BuOH and EtOAc-Pp fractions inhibited MAYV replication in Vero cells. EtOAc and *n*-BuOH fractions inhibited MAYV production, respectively, by more than 70% and 85% at 25 μ g/mL. EtOAc-Pp fraction inhibited MAYV production by more than 90% at 10 μ g/mL. The antiviral ribavirin were much less potent inhibitors of MAYV replication, with IC₉₀ values above 100 μ g/mL (Table 2 and Figure 2).

The Selectivity Index (SI) and the Relative Potency are important indexes that can represent how suitable a substance is for further studies. EtOAc-Pp had the highest SI and 16 times higher Relative Potency than ribavirin. Although this is very relevant data, further studies need to be accomplished in order to address the use of these compounds as antivirals.

Discussion

Since the three fractions tested have phenolic derivatives such as flavonoids and tannins as the major compounds, their antiviral activity can be attributed to the presence of them. EtOAc has flavonoid aglycones and flavonoid monoglycosides as major phenolics compounds, while in *n*-BuOH flavonoid diglycosides are the major ones. For the fraction EtOAc-Pp, beyond flavonoid mono and diglycosides, condensed tannins are present.

The presence of condensed tannins (flavan-3-ol, dimers and trimers) may be one of the factors responsible for antiviral activity. Tannins are known for their property of complexing with proteins, including lipo- and glycoproteins. Previous studies have reported that the binding of polymeric condensed tannins with protein was stronger than that of low molecular weight oligomers and

Table 2 Cytotoxicity and anti-MAYV activity of EtOAc, *n*-BuOH and EtOAc-Pp fractions

Substance	CC ₅₀ (μ g/mL) ^a	CC ₉₀ (μ g/mL) ^a	IC ₅₀ (μ g/mL) ^b	IC ₉₀ (μ g/mL) ^b	SI ^c	RP ^d
<i>n</i> -BuOH	2614 \pm 366	821 \pm 115	7,1 \pm 1,0	40,9 \pm 5,7	20	10
EtOAc	457,7 \pm 9,5	176,1 \pm 3,5	8,2 \pm 0,2	89,1 \pm 4,4	2	1
EtOAc-Pp	324,1 \pm 6,5	154,7 \pm 3,1	2,5 \pm 0,1	4,7 \pm 0,3	33	16,5
ribavirin	523,1 \pm 42,5	215,4 \pm 6,2	62,3 \pm 4,4	112,4 \pm 8,2	2	nd

^a50% and 90% cytotoxic concentration.

^b50% and 90% inhibitory concentration of viral replication.

^cSelectivity Index = standard IC₉₀/substance IC₉₀.

^dRelative Potency = ratio between ribavirin (reference substance) IC₉₀ and the tested substance's IC₉₀.

nd - Not determined.

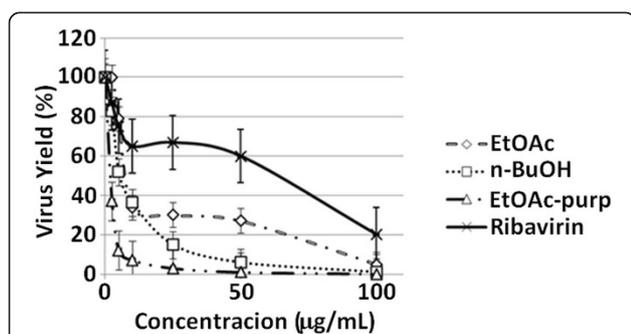


Figure 2 Anti-MAYV activity of different fractions from *C. australis*. The anti-MAYV activity of EtOAc, *n*-BuOH and EtOAc-Pp fractions from *C. australis* was evaluated by treating MAYV-infected cells with 0–100 µg/ml of these fractions for 24 h, and then staining for viral plaque counting. The graph shows the results from three independent experiments. Data are presented as mean% virus yield (compared to untreated controls) ± SD.

monomers. It is believed that hydrogen bonding is an important factor in the binding of condensed tannins gelatin [34].

We know that the effect of the astringent polyphenols, including flavonoids and tannins, is dependent on the affinity of these substances with the protein and due to this is greatly influenced by the composition of each protein, as well as their hydrophilicity, therefore different viruses react polyphenols to different manner. In addition, previous works suggest the tannin-like proanthocyanidins may link the protein covalently [35].

Takechi et al. [36] concluded in his work more highly condensed tannins have a greater antiviral activity, although the galloyl group contributes more to activity than the degree of condensation. It is known that the presence of *o*-dihydroxyphenyl group is related to the formation of protein-polyphenol complex. Moreover, it is thought that tannins interact with the protein particles from the surface of the host cell of the virus, as well as to the viral envelope [36].

In a previous study, Ferreira *et al.* found that the flavonoids quercetin group had a strong antiviral activity against MAYV, suggesting that this virus has proteins that are able to interact with phenolic substances from the group of flavonoid envelope [37].

Condensed tannins have been tested for their antiviral activity and exhibited antiviral activity against respiratory syncytial virus (RSV), influenza A virus (FLU-A) and parainfluenza virus (PIV). It also inhibited the growth of herpes viruses types 1 and 2 (HSV-1, HSV-2) and hepatitis A and B viruses. The proposed mechanism of action was from its connection with the viral envelope proteins, inhibiting the binding and penetration of the virus in the plasma membrane [38].

Yang *et al.* [39] compared several polyphenols derived from tea against influenza A and B and concluded that

condensed tannins were the most active against the influenza A virus than monomeric polyphenols: theaflavin, procyanidin B-2 and procyanidin B-2. To evaluate the structure-activity relationship, they concluded that the dimers as theaflavin and procyanidin B-2, are more active against influenza A and B than the catechin monomers, such as (–)-EC and (±)-catechin and that galoyl group present in theaflavindigallate and procyanidin B-2 digalloyl not help on antiviral effect, probably due to the steric effect [39].

Since the trimers of tannins are only present in EtOAc-Pp, we correlate this to the greater antiviral activity of this fraction. Previous studies have shown that the degree of condensation is an important factor [36,39], being more highly condensed tannins more active; we believe that these substances are responsible for anti-viral activity.

Conclusions

Our results show that *C. australis* is a valuable source of phenolics derivatives with antiviral activity against the arbovirus MAYV. Although antiviral activity of tannins and other phenolics derivatives are very common, this is the first report of anti-MAYV activity for these substances and this species. Our data are an important step in the evaluation of natural products as sources of novel drugs to be used in combination therapy, to circumvent drug resistance, or to replace currently used antivirals with unwanted cytotoxic effects.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RMK and DF conceived and designed the study. AS collected plant material with KCWS. KCWS performed all phytochemical experiments and wrote the initial draft of the manuscript. TSS performed cytotoxicity and viral yield inhibition assays. WR made the ESI(-)FT-ICR MS analyses. MDFM, RC and NKS revised the data and carried out statistical analyses. KCWS, RMK and DF provided invaluable discussions on the chemical data and antiviral chemistry. All authors read and approved the final manuscript.

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