

## ORIGINAL ARTICLE

**Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR**M.C. Búfalo<sup>1</sup>, A.S. Figueiredo<sup>1</sup>, J.P.B. de Sousa<sup>2</sup>, J.M.G. Candeias<sup>1</sup>, J.K. Bastos<sup>2</sup> and J.M. Sforcin<sup>1</sup><sup>1</sup> Department of Microbiology and Immunology, Biosciences Institute, UNESP, Botucatu, SP, Brazil<sup>2</sup> Department of Pharmaceutical Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, Laboratory of Pharmacognosy, USP, Ribeirão Preto, SP, Brazil**Keywords**antiviral action, *Baccharis dracunculifolia*, poliovirus, propolis, real-time PCR.**Correspondence**

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**Abstract****Aims:** The aim of this work was to evaluate the antiviral activities of *Baccharis dracunculifolia* (extract and essential oil), propolis and some isolated compounds (caffeic and cinnamic acids) against poliovirus type 1 (PV1) replication in HEP-2 cells.**Method:** Three different protocols (pre-, simultaneous and post-treatments) were used to verify the effect of addition time of the variables on PV1 replication by crystal violet method and relative viral RNA quantification by real-time PCR for analysing in which step of virus replication the variables could interfere.**Conclusions:** Data revealed that the *B. dracunculifolia* showed the best antiviral activity percentage in the simultaneous treatment, as well as lower relative viral quantification by real-time PCR. Variables might block partially the viral entry within cells, affect the steps of viral cycle replication into cells, or lead to RNA degradation before the virus entry into cells or after their release to the supernatant.**Significance and Impact of the Study:** *Baccharis dracunculifolia* is the most important botanical source of the south-eastern Brazilian propolis, and its potential for the development of new phytotherapeutic medicines has been investigated. Propolis is commonly used for its antimicrobial and immunomodulatory activities. Nevertheless, *B. dracunculifolia* and propolis effects on PV1 have not been investigated yet.**Background**

A variety of natural products and their derivatives have been considered as potential candidates for the treatment of human viral diseases (Huleihel and Isanu 2002; Gekker *et al.* 2005). Recently, the inhibitory effects of medicinal plants extracts on the replication of several viruses have been reported. Herpes simplex virus (HSV), human immunodeficiency virus (HIV), hepatitis B virus (HBV) and severe acute respiratory syndrome (SARS) virus were strongly inhibited by various plants extracts (Mukhtar *et al.* 2008).

Poliovirus (PV) is a single-stranded RNA, nonenveloped virus, belonging to the family Picornaviridae and genus *Enterovirus*. It is the aetiological agent of polio-

myelitis, and once it reaches the central nervous system, one may develop paralytic poliomyelitis – a disease characterized by a classic manifestation of flaccid paralysis. The incidence of paralytic poliomyelitis has been reduced over the last decades, especially by the systematic use of vaccines; however, this disease is still endemic in Asia and Africa (Felipe *et al.* 2006). Because of its replication in several types of cultured cells, PV is one of the most studied and understood viral models (Faccin *et al.* 2007).

As to PV, extracts of *Tridax procumbens*, *Carissa carandas*, *Mallotus philippensis*, *Agaricus brasiliensis* and *Euphorbia grantii* among others were found to be efficient against the replication of poliovirus type 1 (PV1) (Semple *et al.* 2001; Felipe *et al.* 2006). A potent activity against HIV and HSV *in vitro* was observed using extracts of

*Baccharis trinervis* (Palomino *et al.* 2002). In this study, we evaluated the effect of *Baccharis dracunculifolia* against PV1 replication.

*Baccharis dracunculifolia* is used in folk medicine as an anti-inflammatory agent and for the treatment of gastrointestinal diseases. This plant is the most important botanical origin of Brazilian propolis, called green propolis because of its colour (Bankova *et al.* 1999; Da Silva Filho *et al.* 2004).

Propolis is the generic name for the resinous substance produced by honeybees and commonly used to improve health and to prevent several diseases. It has been used for medicinal purposes since ancient times, and its antimicrobial, antitumoural and immunomodulatory activities have been reported (Sforcin 2007). With regard to antiviral properties of propolis, its inhibitory effect on several viruses including influenza, HIV, HSV, adenovirus and vesicular stomatitis virus has been well documented (Ito *et al.* 2001; Gekker *et al.* 2005; Moreno *et al.* 2005). These findings have indicated the potential of propolis as a possible antiviral drug. However, antiviral effects of *B. dracunculifolia* have not been investigated yet.

In this study, the antiviral activity of *B. dracunculifolia* (extract and essential oil), propolis and isolated compounds (caffeic and cinnamic acids) was assessed using PV1. The variables were incubated with HEp-2 cells prior, simultaneously or subsequently, to PV1 addition to the cell cultures in order to understand their mechanism of action.

## Materials and methods

### Plant material, oil and extract isolation and major compounds

*Baccharis dracunculifolia* leaves were collected in ten different regions of Brazil (São Paulo, Minas Gerais and Paraná State) in their natural habitat, between May 2004 and April 2005. Plants were identified by Dr Nelson Ivo Matzenbacher (Department of Bioscience, PUC/RS, Brazil), and voucher specimens ( $n = 1298$ ) were deposited in the Herbarium of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), University of Campinas, São Paulo, Brazil.

Extracts of *B. dracunculifolia* were obtained from dried and powdered leaves samples (500 mg), dissolved in 20 ml of 90% ethanol in 125 ml Erlenmeyer flasks, which were stirred at 40°C and 170 rev min<sup>-1</sup> on a shaker (Innova 4300; New Brunswick Scientific, Edison, NJ). After 2 h, flasks were cooled down to room temperature and filtered using analytical filter papers. Aliquots (5 ml) of the hydroalcoholic extracts were transferred to an appropriate vial and dried under air circulation (40°C). The yielding of crude extracts from *B. dracunculifolia* (90 mg) was of 18%.

The essential oil of the dried leaves (500 g) was extracted by hydro-distillation using a Clevenger-type apparatus. After extraction, the volume of essential oil obtained was measured, and the essential oil was stored in hermetically sealed glass containers with rubber lids, covered with aluminium foil to protect the contents from light and kept under refrigeration at 8°C. The oil yield was 0.6% based on the dry weight of the plant. High performance liquid chromatography (HPLC) profile of *B. dracunculifolia* extract and flame ionization detector (FID) capillary gas chromatogram of the essential oil were carried out (Sousa *et al.* 2007).

Caffeic and cinnamic acids were purchased from Acros Organics (Morris Plains, NJ, USA). Extract, caffeic and cinnamic acids were diluted in 100 µl of 70% ethanol and subsequently diluted in minimum essential medium (MEM; GIBCO, NY, USA) supplemented with 0.1 g l<sup>-1</sup> of L-glutamine, 2.2 g l<sup>-1</sup> sodium bicarbonate, 10 ml l<sup>-1</sup> nonessential amino acids and 10% foetal calf serum (LGC, Biotecnologia LTDA., Cotia, Brazil). Essential oil (1 ml) was diluted in culture medium containing 0.2% dimethylsulfoxide (DMSO; Sigma-Aldrich, USA).

Specific dilutions of each variable (*Baccharis* extract and essential oil, caffeic and cinnamic acids) were prepared for each assay in order to achieve 5, 10, 25, 50 and 100 µg per 100 µl. The same procedure was carried out with 0.2% DMSO (essential oil solvent) and 70% ethanol (extract and isolated compounds solvent) to obtain the respective concentrations of DMSO in the essential oil, and of alcohol in the extract, caffeic and cinnamic acids.

### Propolis sample

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu, Brazil. Propolis was ground, and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 ml with 70% ethanol), in the absence of bright light, at room temperature with moderate shaking. After a week, extracts were filtered, and the dry weight of the extracts was calculated (120 mg ml<sup>-1</sup>). Propolis chemical composition was investigated using thin layer chromatography (TLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses (Bankova *et al.* 1998). Propolis was diluted in MEM, and specific dilutions were prepared to achieve 5, 10, 25, 50 and 100 µg per 100 µl. The same procedure was carried out with 70% ethanol (propolis solvent).

### Cells and virus

HEp-2 cells (human laryngeal epidermoid carcinoma cells) were stored in liquid nitrogen. Afterwards, cells were

cultured in flasks with MEM supplemented with 10% foetal calf serum and, before use, 2 ml of trypsin (0.2% trypsin in 5% EDTA) was added to each flask until cells detachment. Cells were counted using a haemocytometer and cultured in a 96-well flat-bottomed plate (Corning, NY, USA), at a final concentration of  $2 \times 10^5$  cells per well.

PV1 (vaccinal strain Sabin I, stored at  $-80^\circ\text{C}$  in our laboratory) was propagated in HEp-2 cell cultures and maintained at  $-80^\circ\text{C}$ . The virus titre was determined according to Reed and Muench method (Lennette 1995) and expressed in 50% tissue culture infectious dose (TCID<sub>50</sub>).

### Cytotoxic assay

Prior to antiviral assays, cell viability after incubation with each variable in different concentrations was assessed in order to carry out the assays only with noncytotoxic concentrations. The evaluation of cytotoxicity was carried out by crystal violet method (Ait-Mbarek *et al.* 2007).

HEp-2 cells grown in 96-well flat-bottomed microplates were incubated with different concentrations (5, 10, 25, 50 and 100  $\mu\text{g}$  per 100  $\mu\text{l}$ ) of *B. dracunculifolia* (extract and essential oil), propolis, caffeic and cinnamic acids, as well as with 70% ethanol and 0.2% DMSO. Control cells were incubated with medium alone. The final volume in each well was 100  $\mu\text{l}$ .

Cell viability was determined after 48-h incubation at  $37^\circ\text{C}$  and 5% CO<sub>2</sub>. The medium was removed, and 100  $\mu\text{l}$  of 0.5% crystal violet solution was added to the cells. After 10 min of incubation at room temperature, plates were washed, and viable crystal violet-stained cells were lysed with 1% sodium dodecyl sulphate (SDS). Optical densities (OD) were read at 492 nm in an ELISA reader, and the percentage of cell viability was calculated using the formula: (OD test/OD control)  $\times$  100. Assays were carried out in triplicate.

### Antiviral assays

The antiviral effect of the variables on PV1 replication was determined according to Faccin *et al.* (2007) with minor modifications, in three different protocols, as follows:

(i) *Pretreatment*: HEp-2 cells were resuspended at a concentration of  $2 \times 10^6$  cells ml<sup>-1</sup> in MEM, cultured in 96-well flat-bottomed plates and incubated with the variables at noncytotoxic concentrations: *B. dracunculifolia* (extract and essential oil), caffeic and cinnamic acids = 5, 10 and 25  $\mu\text{g}$  per 100  $\mu\text{l}$ ; propolis = 5 and 10  $\mu\text{g}$  per 100  $\mu\text{l}$ . After 2 h, the medium was removed, and 100 TCID<sub>50</sub> per 100  $\mu\text{l}$  (using the dilution of  $10^{-2.5}$  of virus stock) were added and incubated for 48 h at  $37^\circ\text{C}$  and 5% CO<sub>2</sub>.

(ii) *Simultaneous treatment*: HEp-2 cells ( $2 \times 10^6$  cells ml<sup>-1</sup>) were incubated simultaneously with noncytotoxic concentrations of each variable and 100 TCID<sub>50</sub> per 100  $\mu\text{l}$  ( $10^{-2.5}$ ) of virus suspension for 48 h.

(iii) *Post-treatment*: HEp-2 cells ( $2 \times 10^6$  cells ml<sup>-1</sup>) were incubated with 100 TCID<sub>50</sub> per 100  $\mu\text{l}$  ( $10^{-2.5}$ ) of virus suspension for 2 h for virus adsorption. After incubation, the medium was removed, and variables at noncytotoxic concentrations were added and incubated for 48 h.

Cells with virus but without variables were considered as a positive control. Each variable was evaluated in triplicate, and assays were repeated three times. The percentage of antiviral activity, corresponding to viable HEp-2 cells, was assessed by crystal violet method as described above.

In these same conditions, other plates were prepared for relative viral RNA quantification by real-time PCR in order to verify in which step of virus replication the variables could interfere.

After incubation with *B. dracunculifolia* (extract and essential oil), propolis, caffeic and cinnamic acids, 100  $\mu\text{l}$  of supernatant were collected and centrifuged at 400 g for 15 min to remove cellular debris. Afterwards, the cell monolayer was treated with 100  $\mu\text{l}$  of lysis buffer of the RNAspin Mini RNA Isolation kit (GE Healthcare, NJ, USA), and the lysate was collected. Samples of supernatant and cell lysates were submitted to total RNA extraction.

### Extraction of total RNA and cDNA synthesis

Total RNA was extracted using the RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's instructions. Extracted RNA preparations were stored at  $-80^\circ\text{C}$ .

Total RNA extracted (4  $\mu\text{l}$ ) was reverse transcribed with 1  $\mu\text{l}$  of random primer (250 ng  $\mu\text{l}^{-1}$ ), and the mixture was incubated for 5 min at  $70^\circ\text{C}$ . For each sample, the master mix was prepared with 4  $\mu\text{l}$  of reaction buffer (Improm II 5 $\times$ ; Promega, WI, USA), 2.4  $\mu\text{l}$  of MgCl<sub>2</sub> (25 mmol l<sup>-1</sup>), 1  $\mu\text{l}$  of RNase out (10 units), 0.5  $\mu\text{l}$  of dNTP (20 mmol l<sup>-1</sup>), 0.5  $\mu\text{l}$  Improm RT II (Promega) and 6.6  $\mu\text{l}$  of free nuclease water. Afterwards, samples were cooled down to  $4^\circ\text{C}$ , and 15  $\mu\text{l}$  of master mix were added, incubating for 5 min at  $25^\circ\text{C}$ , 60 min at  $42^\circ\text{C}$  and for 15 min at  $70^\circ\text{C}$ . Each cDNA was stored at  $-20^\circ\text{C}$ .

### Real-time PCR

Each tube of reaction contained 4  $\mu\text{l}$  of PV cDNA template, 10  $\mu\text{l}$  of the master mix SYBR Green I (Invitrogen, CA), 0.4  $\mu\text{l}$  of each primer (final concentration

200 nmol l<sup>-1</sup>), 0.4 µl ROX reference dye and 5.2 µl of PCR-grade water. Specific primers for PV capsid gene (VP1–VP4) were 5'-AGT TTC ACC GAA GGC GGA-3' (F) and 5'-CGC TGA CAC AAA ACC AAG GA-3' (R) (GeneBank accession no. AY184219), resulting in a 102-bp amplified product. The PCR programme consisted of the following steps: 95°C for 10 min for initial denaturation, amplification for 40 cycles (95°C for 15 s for denaturation, 60°C for 1 min for annealing and extension), and to confirm the PCR product one cycle of melting curve analysis at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Fluorescence data were collected during each annealing/extension step and analysed using ABI PRISM® 7300 SEQUENCE DETECTOR (Applied Biosystems, CA, USA) and SDS ver. 1.2.3 (Sequence Detection Systems 1.2.3, 7300 Real Time PCR System; Applied Biosystems) software.

In every PCR run, negative (no template) and positive PV controls were processed as a routine quality control of the assay. Assays were carried out in duplicate, and the RNA quantification reduction percentage in the supernatant and lysate samples was calculated considering the positive control as 100%, using the formula: [1 - (RNA samples/RNA control)] × 100.

#### Standard curve

The standard curve was generated by performing serial dilutions of the PV1 RNA extracted of cell culture. To the smallest dilution of RNA standard, it was given the relative value 100 and, following the same reason of dilution, the other 3 points were 50, 25 and 12.5. The virus quantity in the samples was expressed in relation to the standard curve.

#### Statistics

Wilcoxon test was used to detect differences between propolis concentrations, and Friedman test to the other variables, with the significant level at  $P < 0.05$  (Zar 1999).

## Results

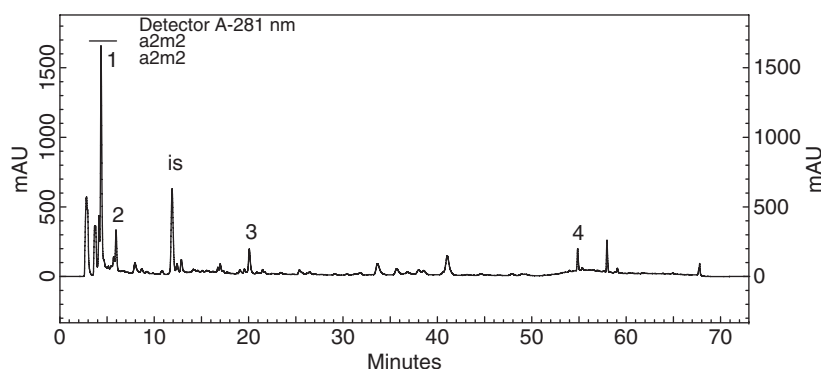
### *Baccharis dracunculifolia* (extract and essential oil) and propolis chemical composition

The chromatographic profiles of *B. dracunculifolia* leaves (extract and essential oil) are showed in Figs 1 and 2, respectively. From HPLC analysis and comparatives studies, involving authentic standard and the samples investigated here, it was possible to carry out the characterization of the extract of this plant. According to Fig. 1, caffeic acid, ferulic acid, aromadendrin-4'-methyl ether and artepillin C were the main phenolics detected in the plant hydroalcoholic extract (Sousa *et al.* 2007). GC-MS and FID capillary gas chromatography analysis of the essential oil of *B. dracunculifolia* revealed that the main chemical components were  $\alpha$ -pinene,  $\beta$ -pinene, limonene, trans-caryophyllene, aromadendrene,  $\alpha$ -humulene, germacrene D, bicyclogermacrene,  $\delta$ -cadinene, nerolidol, spathulenol, viridiflorol, guaiol and  $\alpha$ -muurolol (Fig. 2).

The main constituents of our propolis sample, investigated by TLC, GC and GC-MS analyses, were flavonoids (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether), a prenylated *p*-coumaric acid and two benzopyranes (*E* and *Z* 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-benzopyranes), essential oils (spathulenol, (2*Z*,6*E*)-farnesol, benzyl benzoate and prenylated acetophenones), aromatic acids (dihydrocinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, 3,5-diprenyl-*p*-coumaric acid, 2,2-dimethyl-6-carboxyethenyl-8-prenyl-2H-1-benzo-pyran), di- and tri-terpenes, among others (Bankova *et al.* 1998).

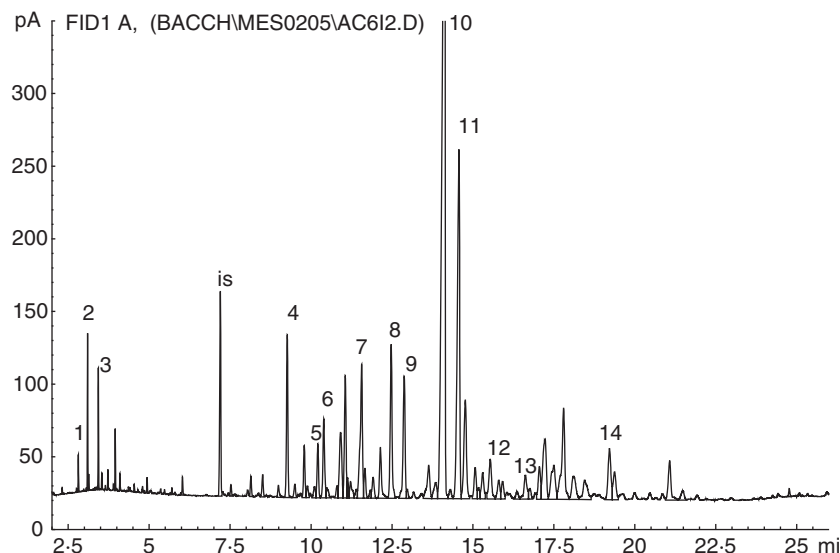
#### Cytotoxic assay

Data showed that the noncytotoxic concentrations for each variable were *B. dracunculifolia* (extract and essential oil), caffeic and cinnamic acids = 5, 10 and 25 µg per 100 µl, and propolis = 5 and 10 µg per 100 µl. Ethanol



**Figure 1** High performance liquid chromatography profile of *Baccharis dracunculifolia* extract: caffeic acid (1), ferulic acid (2), aromadendrin-4'-methyl ether (3) and artepillin C (4), internal standard (is) (veratraldehyde).

**Figure 2** FID capillary gas chromatogram of essential oil of *Baccharis dracunculifolia*:  $\alpha$ -pinene (1),  $\beta$ -pinene (2), limonene (3), trans-caryophyllene, (4), aromadendrene (5),  $\alpha$ -humulene (6), germacrene D (7), bicyclogermacrene (8),  $\delta$ -cadinene (9), nerolidol (10), spathulenol (11), viridiflorol (12), guaiol (13),  $\alpha$ -muurolol (14) and internal standard (is) (piperonal).



and DMSO had no effects in cell viability (Búfalo *et al.* in press).

#### Antiviral assay

For the antiviral assays, noncytotoxic concentrations of each variable were used. In the pretreatment, *B. dracunculifolia* extract showed the most efficient antiviral action (31.0%), followed by *B. dracunculifolia* oil (28.9%), propolis (10.9%), cinnamic acid (10.5%) and caffeic acid (8.5%). With regard to the concentrations of each variable, the most efficient concentration for the plant extract and essential oil was 25  $\mu\text{g}$  per 100  $\mu\text{l}$  in comparison with the other concentrations for the same variable ( $P < 0.05$ ). No significant differences were seen between the different concentrations of propolis, caffeic and cinnamic acids ( $P > 0.05$ ) (Figs 3a–7a).

In the simultaneous treatment, the highest inhibition of virus replication was achieved by *Baccharis* extract (74.0%), followed by propolis (52.2%), essential oil (33.3%), cinnamic acid (29.8%), and caffeic acid (26.7%). The most efficient concentration for *Baccharis* extract, caffeic and cinnamic acids was 25  $\mu\text{g}$  per 100  $\mu\text{l}$ , followed by propolis (10  $\mu\text{g}$  per 100  $\mu\text{l}$ ) ( $P < 0.05$ ). There were no significant differences between the different concentrations of *Baccharis* essential oil ( $P > 0.05$ ) (Figs 3b–7b).

Data from the post-treatment showed that the most efficient variables were *Baccharis* essential oil (49.6%), propolis (39.1%), *Baccharis* extract (36.1%), caffeic acid (22.3%) and cinnamic acid (7.9%). As to the concentrations, *Baccharis* (extract and essential oil) and caffeic acid (25  $\mu\text{g}$  per 100  $\mu\text{l}$ ) and propolis (10  $\mu\text{g}$  per 100  $\mu\text{l}$ ) inhibited significantly the PV1 replication ( $P < 0.05$ ). No sig-

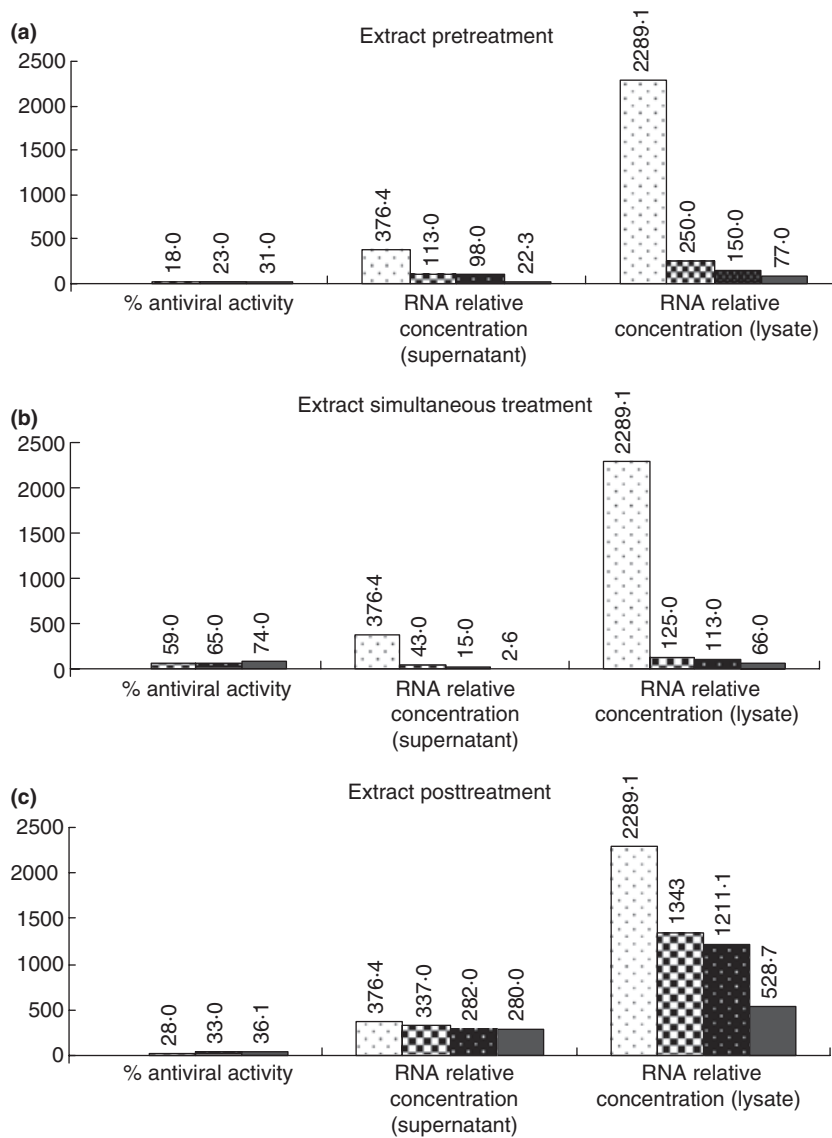
nificant differences were seen between the concentrations of the cinnamic acid ( $P > 0.05$ ) (Figs 3c–7c).

A comparison between the variables regarding their concentrations and antiviral action in all protocols (pre-, simultaneous and post-treatments) revealed that *Baccharis* extract (25  $\mu\text{g}$  per 100  $\mu\text{l}$ ) showed the best antiviral activity (74.0%) in the simultaneous treatment ( $P < 0.05$ ) (Table 1).

#### Relative quantification of viral RNA

PCR efficiency (78.1%) was determined using the slope of the standard curves, and the real-time PCR reproducibility was represented by the correlation coefficient ( $R = 0.99$ ). Amplicons specificity was confirmed by melting curve analysis. A single melting peak at 80°C to specific amplicon of 102 bp was obtained, indicating that the primers used in this study were specific for PV1 (data not shown).

In the pretreatment, the highest RNA quantification reduction percentage was seen in the supernatant samples after incubation with *B. dracunculifolia* extract, caffeic and cinnamic acids, in comparison with the positive controls (94%, 90% and 89% respectively), using 25  $\mu\text{g}$  per 100  $\mu\text{l}$ . These same variables also showed higher RNA reduction percentage than the essential oil and propolis, using 10 and 5  $\mu\text{g}$  per 100  $\mu\text{l}$  (*Baccharis* extract = 74% and 70%; caffeic acid = 79% and 73%; cinnamic acid = 78% and 65% respectively). *Baccharis* essential oil showed the following RNA reduction percentages 66%, 64% and 63%, using 25, 10 and 5  $\mu\text{g}$  per 100  $\mu\text{l}$ . For propolis, the reduction percentages were 60% and 41%, using 10 and 5  $\mu\text{g}$  per 100  $\mu\text{l}$  (Figs 3a–7a).



**Figure 3** Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the extract. (a): Pretreatment, (b): simultaneous treatment, (c): post-treatment. Friedman test ( $P < 0.05$ ). (▨) 0 µg per 100 µl; (▩) 5 µg per 100 µl; (▤) 10 µg per 100 µl and (■) 25 µg per 100 µl.

In cell lysates, *B. dracunculifolia* extract showed the best RNA reduction percentage in all concentrations (97%, 93% and 89%), followed by the essential oil, propolis, caffeic and cinnamic acids (Figs 3a–7a).

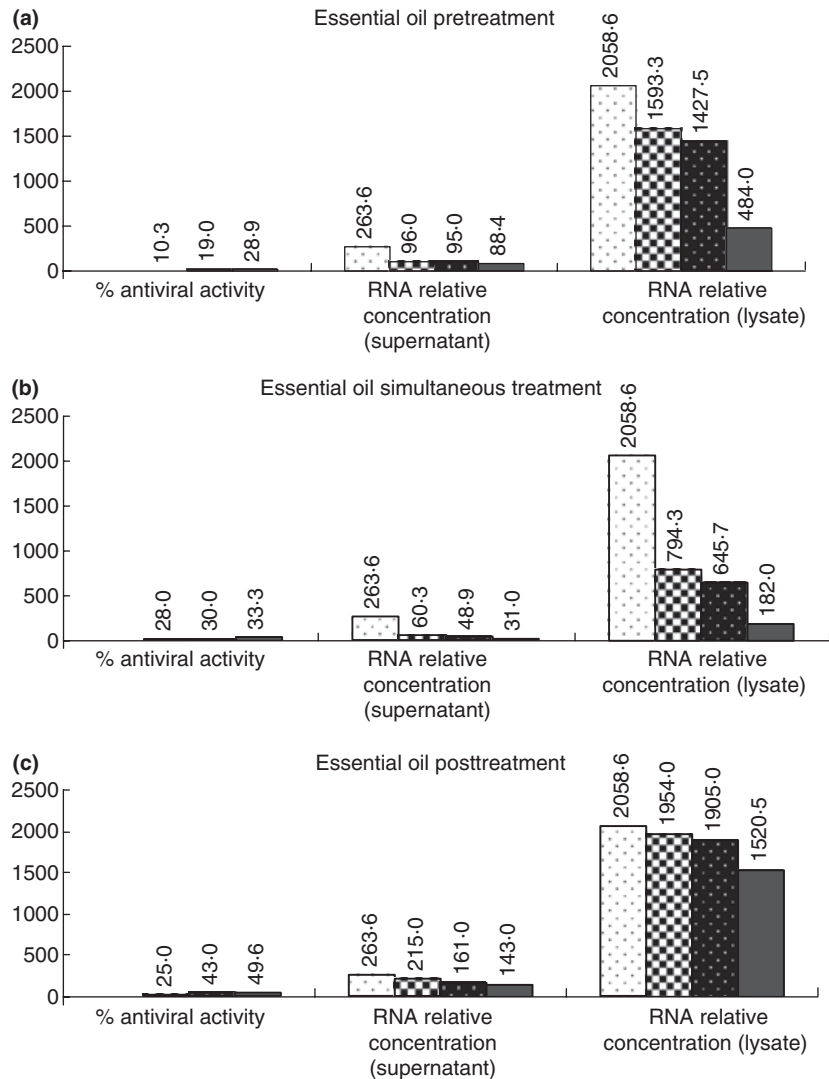
In the simultaneous treatment, the highest RNA quantification reduction percentage was seen in the supernatant samples after incubation with *B. dracunculifolia* extract (99%, 96% and 89%, respectively to 25, 10 and 5 µg per 100 µl), followed by cinnamic acid (93%, 88% and 72%), caffeic acid (93%, 80% and 72%) and *Baccharis* essential oil (88%, 81% and 77%). For propolis, the RNA reduction percentages were 80% and 30%, using 10 and 5 µg per 100 µl respectively (Figs 3b–7b).

In cell lysates, *Baccharis* extract led to decreased RNA quantification (RNA reduction percentages: 97%, 96% and 95%, using 25, 10 and 5 µg per 100 µl). The RNA

reduction percentages for the other variables were: *Baccharis* essential oil = 91%, 69% and 61%; cinnamic acid = 85%, 46% and 18%; caffeic acid = 85%, 45% and 23%; and propolis = 77% and 68% (Figs 3b–7b).

In the post-treatment, caffeic acid led to the lowest relative viral RNA in the supernatant (RNA reduction percentage: 76%, 51% and 44%), followed by the other variables (cinnamic acid = 72%, 48% and 40%; propolis = 53% and 20%; essential oil = 46%, 39% and 18%; *Baccharis* extract = 26%, 25% and 10%) (Figs 3c–7c).

In cell lysates, the RNA reduction percentages were: *Baccharis* extract = 77%, 48% and 41%; cinnamic acid = 58%, 15% and 14%; propolis = 35% and 3%; essential oil = 26%, 8% and 5%, and caffeic acid = 18%, 12% and 13% (Figs 3c–7c).



**Figure 4** Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the essential oil. (a): Pretreatment, (b): simultaneous treatment, (c): post-treatment. Friedman test ( $P < 0.05$ ). (▨) 0 µg per 100 µl; (▩) 5 µg per 100 µl; (▤) 10 µg per 100 µl and (■) 25 µg per 100 µl.

The best results were obtained in the simultaneous treatment, followed by the pre- and post-treatments for *B. dracunculifolia* extract, caffeic and cinnamic acids, *Baccharis* essential oil and propolis, either in the supernatants or in cells lysates. Higher relative viral RNA quantification was observed in the cell lysate in comparison with the supernatants, in all protocols and variables (Figs 3–7).

One may verify that real-time PCR data are in agreement with the Table 1, and an association between the highest antiviral activities of the variables in the crystal violet method could be established with the lowest relative viral quantification in the real-time PCR.

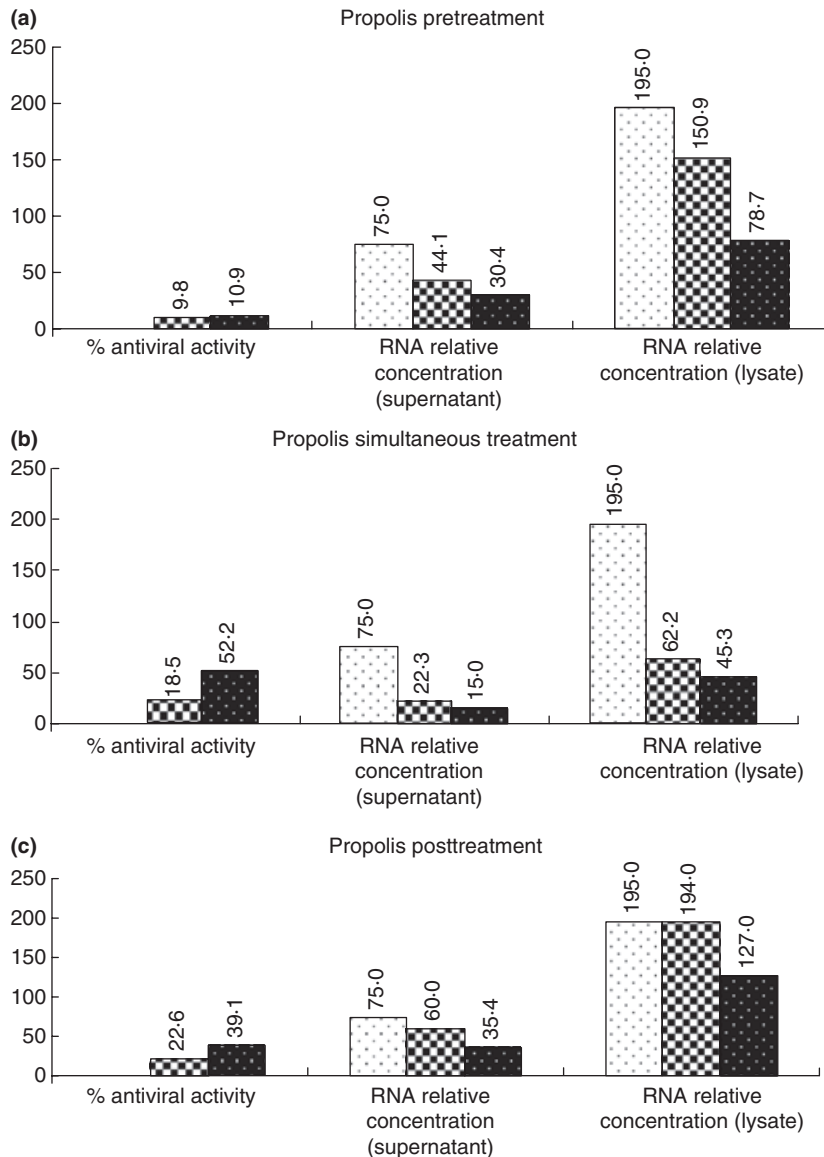
**Discussion and conclusions**

Medicinal plants have been widely used to treat a variety of infectious and noninfectious diseases, and 25% of the commonly used medicines contain compounds isolated

from plants (Mukhtar et al. 2008). The investigation of natural products with antiviral action has attracted the researchers’ interest; nevertheless, no articles are found in the literature dealing with antiviral activity of *B. dracunculifolia*. In this study, we wish to report for the first time the antiviral activity of this plant on PV1 replication.

With regard to the antiviral assays, the highest percentage of viral inhibition and consequently smallest relative RNA viral quantification were obtained with 25 µg per 100 µl of *B. dracunculifolia* extract in the simultaneous treatment. *Baccharis dracunculifolia* essential oil showed a lower antiviral activity compared to the extract.

When PV1 was added to the cells simultaneously with the extract or propolis, there was a decreased RNA quantification in cell lysate as well as in the supernatant samples, however, higher amounts of RNA were found in the lysate in comparison with the supernatant. Although the viral entry into cells could have been inhibited,



**Figure 5** Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the propolis. (a): Pretreatment, (b): simultaneous treatment, (c): post-treatment. Wilcoxon test ( $P < 0.05$ ). (▨) 0 µg per 100 µl; (▩) 5 µg per 100 µl and (■) 10 µg per 100 µl.

*B. dracunculifolia* and propolis exerted their antiviral activity probably when PV1 was within cells, affecting the cycle replication.

In the pretreatment, variables were removed before adding the virus, what could lead cells more resistant to virus attack. However, there was a higher viral entry into cells, in comparison with the simultaneous treatment, discarding our hypothesis. On the other hand, in the post-treatment, variables were added after the virus, and the RNA viral quantification was higher than that in the simultaneous treatment. One may speculate that variables interfered in virus output by infected cells, or led to RNA degradation in the supernatant after virus output.

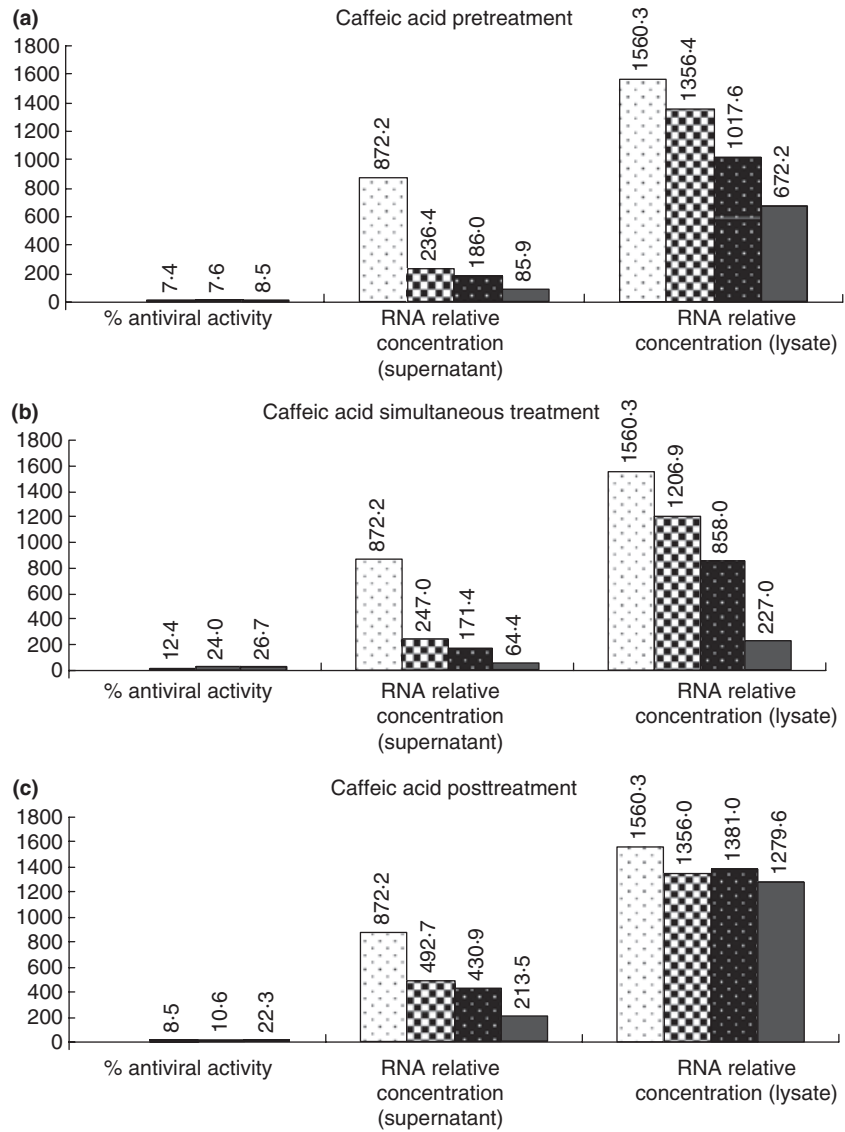
Extracts of *B. trinervis* showed inhibitory effects on HSV and HIV replication when added simultaneously to

the virus, suggesting that the extracts inhibited the virus-cell attachment, virus-cell fusion and cell-to-cell fusion (Palomino *et al.* 2002).

As to propolis, Amoros *et al.* (1992) verified the effect *in vitro* of alcoholic extract of propolis against several viruses of DNA and RNA, including HSV, adenovirus, and vesicular stomach virus, showing that propolis reduced HSV titres; but the other viruses were less susceptible to its action.

Serkedjieva *et al.* (1992) reported that the pretreatment of canine kidney epithelial (MDCK) cells with propolis had no effect on influenza virus replication. However, a reduced viral infectivity was seen adding propolis simultaneously with the virus or immediately after virus adsorption, suggesting that adsorption and the penetration of





**Figure 6** Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the caffeic acid. (a): Pretreatment, (b): simultaneous treatment, (c): post-treatment. Friedman test ( $P < 0.05$ ). (□) 0 µg per 100 µl; (▨) 5 µg per 100 µl; (▩) 10 µg per 100 µl and (■) 25 µg per 100 µl.

virus were inhibited in the initial stage of replication cycle.

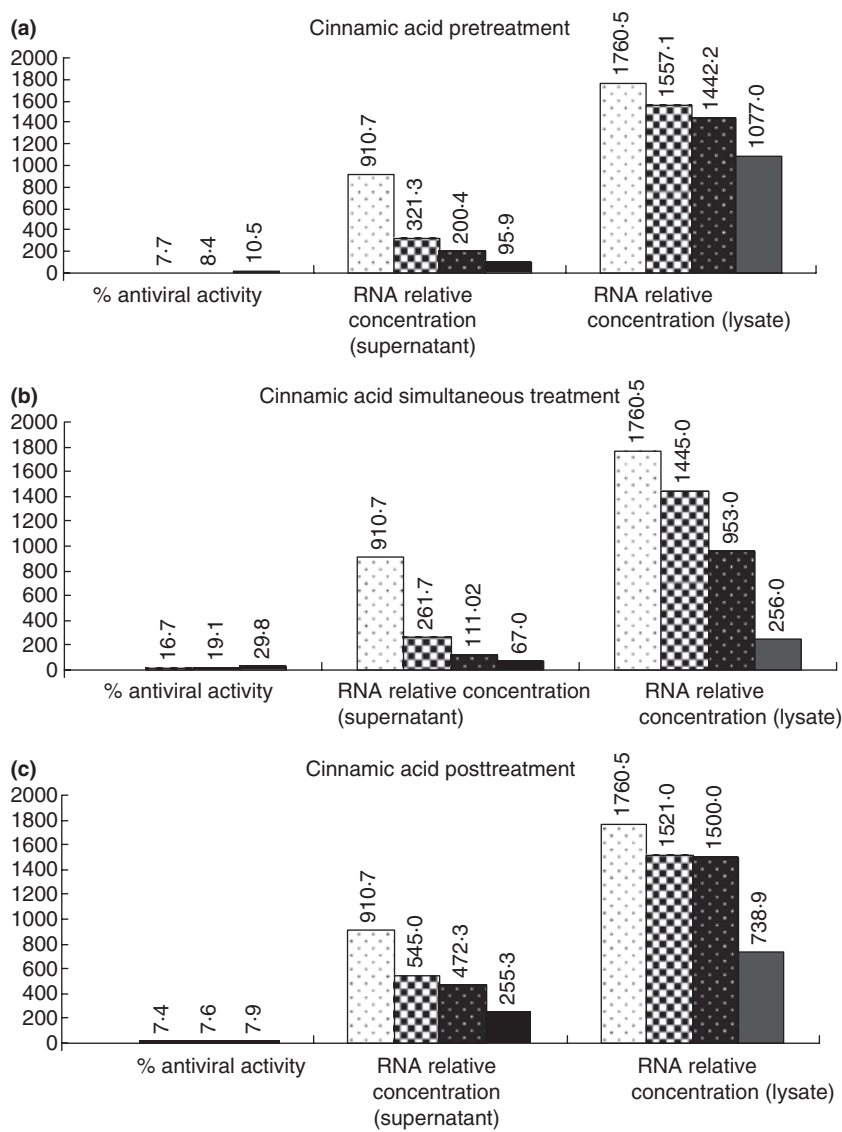
Ito *et al.* (2001) verified the efficient activity of propolis anti-HIV *in vitro*. Gekker *et al.* (2005), using microglial cell cultures, showed propolis activity against HIV in a concentration-dependent manner, suggesting that this effect may have occurred because of caffeic acid phenethyl ester (CAPE) action, involving, in part, the inhibition of viral entry into cells.

According to Huleihel and Isanu (2002), propolis caused 50% inhibition of HSV infection, and indirect evidence pointed out to a strong interaction between propolis and the surface of Vero cells, but not with HSV particles. Administration of propolis before or simultaneously to infection yielded the most significant

inhibitory effect, suggesting that this effect were because of propolis blockage of the cell membrane receptors for HSV or because of changes inside the cells, which could in turn affect the virus replication cycle.

Huleihel and Ishano (2001) and Matsuo *et al.* (2005) suggested that the antiviral activity of propolis might be attributed to flavonoids action, which play a significant role in the antiviral process. Tait *et al.* (2006) related that natural and synthetic flavonoids might interfere with picornavirus replication preventing the decapsidation of viral particles and RNA release within cells or blocking viral RNA synthesis.

Isolated compounds are important to understand the possible mechanism of action of propolis and its vegetal source. In our study, caffeic and cinnamic acids showed a



**Figure 7** Antiviral activity percentage and relative RNA viral quantification (supernatant and cell lysate) of the cinnamic acid. (a): Pretreatment, (b): simultaneous treatment, (c): post-treatment. Friedman test ( $P < 0.05$ ). (▨) 0 µg per 100 µl; (▩) 5 µg per 100 µl; (▤) 10 µg per 100 µl and (■) 25 µg per 100 µl.

**Table 1** Comparison between the protocols (pre-, simultaneous and post-treatments) with regard to the antiviral activity of each variable

Variables	5 µg per 100 µl			10 µg per 100 µl			25 µg per 100 µl		
	Pretreatment	Simultaneous treatment	Posttreatment	Pretreatment	Simultaneous treatment	Posttreatment	Pretreatment	Simultaneous treatment	Posttreatment
Propolis	b	ab	a	b	a	ab	–	–	–
Extract	b	a	ab	b	a	ab	b	<b>a (74.0%)</b>	ab
Essential oil	a	a	a	b	ab	a	b	ab	a
Caffeic acid	a	a	a	b	a	ab	b	a	ab
Cinnamic acid	a	a	a	b	a	b	ab	a	b

Different small letters indicate significant differences between the treatments in the same concentration by Friedman test ( $P < 0.05$ ). The best antiviral activity is shown in bold, and the percentage is between parentheses.

lower antiviral activity when compared to *B. dracunculifolia* and propolis, suggesting that these acids may be involved in *B. dracunculifolia* and propolis' antiviral

effects. It has been reported that the potential of propolis and *Baccharis* are due to a natural mixture of its components, and that a single constituents would not have an

activity greater than that the total extract (Kujumgiev *et al.* 1999).

The real-time PCR has been described as a quantitative detection method for nucleic acids (Min *et al.* 2006). This method provides higher sensitivity and specificity to quantify viral nucleic acids, and an association between the concentrations of viral nucleic acid and cell culture infectivity by PV1 could be established herein.

In our study, *B. dracunculifolia* (25 µg per 100 µl) showed an efficient percentage of antiviral activity evidenced by the crystal violet method (74.0%) in the simultaneous treatment, which was associated to the lowest relative viral quantification in the real-time PCR. Some explanations to these effects may be raised: (i) variables might block partially the viral entry within cells, (ii) variables could have affected the steps of viral cycle replication into cells and (iii) RNA degradation before the virus entry into cells or after virus release to the supernatant. However, more investigations are still needed in order to explore the potential of these variables as antiviral agents and to understand their mechanisms of action against PV1 cycle replication.

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