Short communication

Evaluation of chemical and antiviral properties of essential oils from South American plants

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The essential oils of seven aromatic plants from Córdoba, San Luis and San Juan Provinces (Argentina) were isolated by steam distillation and analysed by a gas chromatography/mass spectrometry technique. The oils were screened for cytotoxicity and *in vitro* inhibitory activity against herpes simplex virus type 1 (HSV-1), dengue virus type 2 (DENV-2) and Junin virus (JUNV) by a virucidal test. The oils showed a variable virucidal action according to the virus. The better relationship between cytotoxicity and antivirus action was observed with the essential oils of *Heterothalamus alienus* and *Buddleja cordobensis* against JUNV, with virucidal concentration 50% (VC_{50}) values of 44.2 and 39.0 ppm and therapeutic indices (cytotoxicity to virucidal activity ratio) of 3.3 and 4.0, respectively. The inhibitory action was exerted by a direct interaction of virions with the oils. Virions inactivated with *B. cordobensis* and *H. alienus* essential oil were not affected in their ability to bind to the host cell. The therapeutic indices shown by these essential oils *in toto* were very modest, but given the complexity of their chemical composition the future identification of the precise active principle may allow the elimination of cytotoxic components and increase the selectivity of the effective compound.

Key words: Junin virus, dengue virus, herpes simplex virus, South American plants, essential oils, virus inactivation

Introduction

Plant products provide an interesting alternative source for the discovery of lead compounds guiding to the identification of novel antiviral agents (Vlietinck *et al*., 1998). Among the structurally diverse plant components, the essential oil fraction, responsible for plant fragance, has started to be analysed for antimicrobial properties in recent years (Cowan, 1999). Particularly, human herpesviruses, such as herpes simplex virus type 1 (HSV-1) and 2 (HSV-2), were described as susceptible to the inhibitory action of plant essential oils (Hayashi *et al*., 1995; Armaka *et al*., 1999; Benencia and Courreges, 1999; De Logu *et al*., 2000; Schnitzler *et al*., 2001; Primo *et al*., 2001; Minami *et al*., 2003; Schuhmacher *et al*., 2003; Farag *et al*., 2004; Vijayan *et al*., 2004; Allahverdiyev *et al*., 2004; Sinico *et al*., 2005).

The present study was aimed to evaluate the antiviral properties of various kinds of essential oils from South American plants. The viruses chosen for inhibitory assays were HSV-1, due to the activity background mentioned above, and two re-emerging RNA viruses lacking any effective chemotherapeutic agent: the arenavirus Junin (JUNV), agent of Argentine haemorrhagic fever (Damonte and Coto, 2002), and dengue virus (DENV), the most important arthropod-borne virus in terms of disease and mortality in tropical and subtropical regions (Gubler, 2002). In a preliminary study, we have reported the presence of a potent virucidal activity in essential oils extracted from leaves of two species of Verbenaceae, *Lippia junelliana* and *Lippia turbinata* (García *et al*., 2003). Here, the analysis was extended to other aromatic plants with unknown essential oil composition and antiviral properties.

Leaves and flowers of *Pectis odorata* Griseb (v.n. cominillo) (Asteraceae) VMA 2791; leaves, flowers and

fruits of *Gaillardia megapotamica* (Spreng) Backer var. radiata (Gris.) Backer (v.n botón de oro) (Asteraceae) VMA 2604; leaves of *Heterothalamus alienus* (Spreng.) Kunze (v.n. romerillo) (Asteraceae) VME 3189; leaves of *Aloysia triphylla* (L'Herit) Britton (v.n. cedrón) (Verbenaceae) VME 2662; leaves of *Artemisia mendozana* D.C. (v.n. ajenjo) (Asteraceae) BT6; leaves of *Jungia polita* Griseb (v.n. zarzaparrilla) (Asteraceae) VMA 2678; leaves and flowers of *Buddleja cordobensis* Griseb (v.n. salvia blanca) (Buddlejaceae) VME 2885, were collected in Córdoba, San Luis, and San Juan Provinces, Argentina. Herbarium specimens were identified and deposited at the Department of Botany, Faculty of Engineering, University of San Luis, Argentina, except *A. mendozana* which was classified and stored at IMBIV in University of Córdoba, Argentina.

Fresh plants were air-dried for 4 days at room temperature. The essential oils, obtained by steam distillation in a Clevenger-type apparatus for 3 h, were dried over anhydrous sodium sulfate, and stored at −18°C in the dark. Gas chromatography (GC) and mass spectrometry (MS) were performed to identify their constituents. The GC analysis was performed on a Hewlett Packard 5890 Series II gas chromatograph with FID detector, equipped with a HP-5 column (5% phenylmethyl silicone, 30 m × 0.25 mm; film thickness 0.25 μm), with nitrogen as carrier gas (1.1 ml/min). The oven was programmed from 75°C (4 min), 75°–135°C (5°C/min), 135°–220°C (1°C/min) and 220°–280°C (10°C/min). Injection volume was 0.1 μl, injection port at 250°C and detector at 270°C. The GC/MS analysis was carried out on a GC-HP 6890 with mass selective detector (quadrupole) HP 5973, source 70ev, fitted with a HP-5MS column (5% phenylmethyl siloxane, 30 m × 0.25 mm; film thickness 0.25 μm). with helium as carrier gas (1.0 ml/min). Injection port at 250°C, MS Source 230°C, MS Quad 150°C, Aux. 280°C. The oven was programmed as above. Injection volume was 0.1 μl, (split 1:80). The identification of oil components was based on comparison of their mass spectra with those found in the literature (Duschatzky *et al.*, 2003), MS data bank (NIST) and a computer search of the Wiley library (McLafferty & Stauffer, 1994). The retention index was determined by co-injection of the homologous N-hydrocarbon C_8-C_{18} mixture with the oil sample. The major components and yield of each oil are listed in Table 1. As seen, several differences in yields and chemical composition from a qualitative and quantitative point of view could be detected when comparing all the analysed oil samples.

For the biological tests, stock solutions of the essential oils were prepared by dissolving each oil in Tween 80-ethanol (1:0.5) and then diluted in maintenance medium (MM: Eagle's minimum essential medium supplemented with 1.5% inactivated calf serum). The cytotoxicity of the essential oils was first investigated by incubating for 24 h subconfluent monolayers of Vero (African green monkey kidney) cells grown in 96-well microplates with serial twofold dilutions of each oil, 3 wells for each concentration. Then cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) method as previously described (García et *al.*, 2000), and the cytotoxic concentration 50% (CC_{50}) was calculated as the oil concentration required to reduce the MTT signal by 50%. As seen in Table 2, the values of CC_{50} were in the range 72–299 ppm.

Based on CC_{50} determinations, the inhibitory activity of the oils against JUNV strain IV4454, DENV type 2 (DENV-2) strain NGC, and HSV-1 strain F was analysed at concentrations lower than 250 ppm by a virucidal test. This assay was chosen based on previous reports on essential oils and the cytotoxicity data shown in Table 2 which discouraged the use of this type of compound as a virus replication inhibitor in infected cells. To this end, a virus suspension containing approximately 10^6 pfu of JUNV, DENV-2 or HSV-1 was incubated with an equal volume of MM with or without serial twofold dilutions of the essential oils for 1.5 h at 37°C. A control virus sample in MM containing the solvent used to prepare the oil solution was also performed. The samples were then diluted in cold MM to determine residual infectivity in a plaque formation assay using Vero cells. This virucidal assay was performed in triplicate. Plaques were developed and counted at 2, 6 and 7 days post-infection for HSV-1, DENV-2 and JUNV, respectively. The ratios of virus titer in compound-treated samples to virus titer in control samples were calculated, and then the virucidal concentration 50% (VC_{50}), defined as the concentration required to reduce virus titer by 50%, was determined. In this assay the virus-oil mixtures were diluted before cell infection to assess lack of toxic effects when the oil sample was in contact with the cell cultures.

Among all the evaluated plants, *A. triphylla* was the only one inactive against the three viruses. The essential oils extracted from the other six plants showed a variable virucidal action according to the virus. In general, most compounds were not very efficient inhibitors of DENV-2 and HSV-1 since the values of VC_{50} were similar to CC_{50}, with only a few exceptions (Table 2). A more effective inhibition was observed against the arenavirus JUNV: of the seven studied plants, four (*P. odorata, G. megapotamica, H. alienus* and *B. cordobensis*) exhibited VC_{50} values lower than 50 ppm. As seen in Table 2, the oils from *H. alienus* and *B. cordobensis* presented the better relationship between cytotoxicity and anti-JUNV activity, and in particular *B. cordobensis* was the only species showing selective virucidal effect against the three tested viruses, with VC_{50} values of 86.4, 54.1 and 39.0 ppm for DENV-2, HSV-1 and JUNV, respectively. However, the oils from *H. alienus*

Table 1. Essential oils: main components and yield

Essential oil	Vernacular name	Main components (%)	Yield (%)
Aloysia triphylla	Cedrón	Alpha tujone 22.9% Cis-carveol 17.51% Carvone 13.24% Limonene 12.72%	3.80
Pectis odorata	Cominillo	Limonene 50.2% Neral 27.2% Geranial 23.6%	1.85
Gaillardia megapotamica	Botón de oro	β-pinene 24.2% Z-β-ocimene 16.5% α-pinene 7.7% Limonene 7.5% β-caryophylene 6.7%	0.30
Heterothalamus alienus	Romerillo	β-pinene 35.5% Sphatulenol 10.7% Germacrene D 6.8%	0.49
Artemisia mendozana	Ajenjo	Camphor 22.41% Artemisole 11.7% Artemisia alcohol 10.8% Borneol 7.2%	0.60
Jungia polita	Zarzaparrilla	Caryophylene oxide 9.18% Trans-caryophylene 8.13%	0.05
Buddleja cordobensis	Salvia blanca	Caryophylene oxide 32.09% β-caryophylene 16.54% α-copaene 8.53%	0.03

Table 2. Cytotoxicity and virucidal activity of essential oils

		VC_{50} (ppm)†		
Essential oil	CC_{50} (ppm)*	DENV-2	JUNV	HSV-1
Aloysia triphylla	201.9 ±22.5	>250	>250	>250
Pectis odorata	73.9 ±5.1	39.6 ±1.8	36.6 ±3.5	71.5 ±0.1
Gaillardia megapotamica	72.2 ±4.2	140.6 ±8.0	49.8 ±0.1	99.1 ±1.0
Heterothalamus alienus	147.6 ±5.7	122.3 ±2.7	44.2 ±7.0	148.4 ±5.1
Artemisia mendozana	298.6 ±15.4	129.3 ±4.3	178.6 ±13.8	153.7 ±28.7
Jungia polita	133.0 ±5.8	39.8 ±.4	134.2 ±9.2	136.4 ±13.3
Buddleja cordobensis	157.2 ±3.5	86.4 ±4.5	39.0 ±3.2	54.1 ±8.4
Lippia turbinata	313.5 ±20.4	>250	14.0 ±0.6	>250

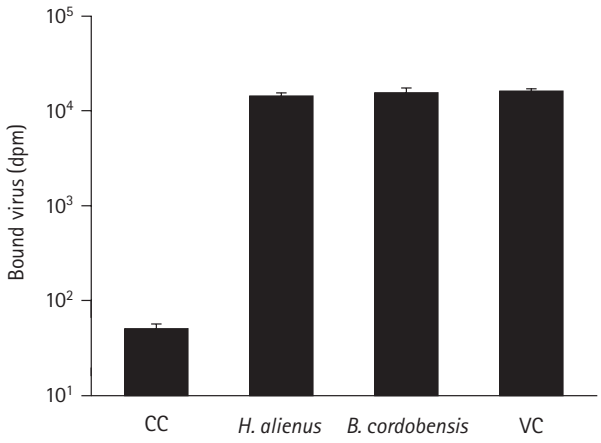
*Cytotoxic concentration 50%: Compound concentration required to reduce cell viability by 50%, determined by MTT method. Values are the mean from triplicate independent tests ± standard deviation. †Virucidal concentration 50%: Compound concentration required to reduce virus titer by 50%. Values are the mean from triplicate independent tests ± standard deviation.

and *B. cordobensis* showed lower potency against JUNV than *Lippia turbinata*, a plant previously studied for virucidal activity in our laboratory (García *et al.*, 2003) and included for comparison in Table 2. A direct inactivating action of virus infectivity was also reported for essential oils obtained from *Santolina insularis* (De Logu *et al.*, 2000), Australian tea tree and eucalyptus (Schnitzler *et al.*, 2001), *Houttuynia cordata* (Hayashi *et al.*, 1995), *Cynbopogon cytratus* (Minami *et al.*, 2003), *Salvia fruticosa* (Sivropoulou *et al.*, 1997), *Mentha piperita* (Schuhmacher *et al.*, 2003), and several *Melaleuca* species (Farag *et al.*, 2004).

To gain some insight into the mechanism by which these compounds affect JUNV infectivity, the ability of virions incubated with *B. cordobensis* and *H. alienus* essential oils to perform the cell adsorption process was determined. To this end, a radiolabelled virion binding assay was performed. The preparation of ^{35}S-methionine-labelled purified JUNV was described previously (Damonte *et al.*, 1994). Radiolabelled virions (2×10^7pfu/ml, 5×10^8 dpm/ml) were mixed with MM or 100 ppm of each essential oil and incubated at 37°C for 1.5 h. After incubation, the viruses were collected by ultracentrifugation and unbound compound was removed. For the binding assay, the viruses were then added to Vero cells grown in a 24-well microplate and incubated at 4°C for 40 min. Then, cells were washed extensively with PBS, lysed with a 0.1 M NaOH solution containing 1% sodium dodecyl sulphate (SDS), and cell-bound radioactivity was quantified using a liquid scintillation counter. Figure 1 shows that the binding to Vero cells of JUNV after treatment with *B. cordobensis* or *H. alienus* compounds was comparable to that of the control virus. Considering the lipophilic nature of the oil which enables it to penetrate membranous structures, the viral envelope may be suspected to be the target of action. Since JUNV virions inactivated with *B. cordobensis* and *H. alienus* essential oil were not affected in their binding ability to Vero cells, a post-adsorption event probably involved in virus entry and controlled by the glycoproteins inserted in the envelope may be affected by the oil treatment.

Although these results support the potential use of essential oils *in toto* as virucidal agents against human viruses, the identification of the precise active principle may allow the elimination of cytotoxic components and increase the selectivity of the effective compound. The GC and mass spectra analyses allowed the identification of more than 40 components in each plant (data not shown), a result indicative of the complexity of their chemical composition. Further studies will be focused on assaying the biological activity of the major components shown in Table 1, in particular with the more active plants such as *H. alienus* and *B. cordobensis*, to improve the performance of these natural products and elucidate their mode of interaction with the virus.

Figure 1. Binding of radiolabelled purified virions of JUNV



Vero cells were incubated during 40 min at 4°C with [^{35}S]-labelled virus samples previously treated with each essential oil at a concentration of 100 ppm or MM for 1.5 h at 37°C, and then cell-bound radioactivity was determined. CC, mock infected cell control; VC, infectious virion control treated with MM; *H. alienus* and *B. cordobensis*, virion samples inactivated with each essential oil. Each value is the mean of duplicate independent experiments ± standard deviation.

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References

Allahverdiyev A, Duran N, Ozguven M & Koltas S (2004) Antiviral activity of the volatile oils of *Melissa officinalis* L against herpes simplex virus type-2. *Phytomedicine* **11:**657–661.

Armaka M, Papanikolau E, Sivropoulou A & Arsenakis M (1999) Antiviral properties of isoborneol, a potent inhibitor of herpes simplex virus type 1. *Antiviral Research* **43:**79–92.

Benencia F & Courreges MC (1999) Antiviral activity of sandalwood oil against herpes simplex viruses-1 and -2. *Phytomedicine* **6:**119–123.

Cowan MM (1999) Plant products as antimicrobial agents. *Clinical Microbiology Reviews* **12:**564–582.

Damonte EB & Coto CE (2002) Treatment of arenavirus infections: from basic studies to the challenge of antiviral therapy. *Advances in Virus Research* **58:**125-155.

Damonte EB, Mersich SE & Candurra NA (1994) Intracellular processing and transport of Junin virus glycoproteins influences virion infectivity. *Virus Research* **34:**317–326.

De Logu A, Loy G, Pellerano ML, Bonsignore L & Schivo ML (2000) Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell spread by *Santolina insularis* essential oil. *Antiviral Research* **48:**177–185.

Duschatzky C, Almeida N, Colombres S & Lampasona M (2003) Essential oil of *Gaillardia megapotamica* (Spreng.) Backer, var. Radiata (Gris.) Backer from San Luis, Argentina. *Journal of Essential Oil Research* **15:**112–113.

Farag RS, Shalaby AS, El-Baroty GA, Ibrahim NA, Ali MA & Hassan EM (2004) Chemical and biological evaluation of the essential oils of different Melaleuca species. *Phytotherapy Research* **18:**30–35.

García CC, Candurra NA & Damonte EB (2000) Antiviral and virucidal activities against arenaviruses of zinc-finger active compounds. *Antiviral Chemistry & Chemotherapy* **11:**231–238.

García CC, Talarico L, Almeida N, Colombres S, Duschatzky C & Damonte EB (2003) Virucidal activity of essential oils from aromatic plants of San Luis, Argentina. *Phytotherapy Research* **17:**1073–1075.

Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends in Microbiology* **10:**100–103.

Hayashi K, Kamiya M & Hayashi T (1995) Virucidal effects of the steam distillate from Houttuynia cordata and its components on HSV-1, influenza virus and HIV. *Planta Medica* **61:**237–241.

McLafferty FW & Stauffer DB (Editors) 1994. *Wiley Registry of Mass Spectral Data*, 6th Ed. Mass Spectrometry Library Search System, Bench-Top PBM, version 3.10d. Palisade: Newfield.

Minami M, Kita M, Nakaya T, Yamamoto T, Kuriyama H & Imanishi J (2003) The inhibitory effect of essential oils on herpes simplex virus type-1 replication *in vitro*. *Microbiology & Immunology* **47:**681–684.

Primo V, Rovera M, Zanon S, Oliva M, Demo M, Daghero J & Sabini L (2001) Determination of the antibacterial and antiviral activity of the essential oil from *Minthostachys verticillata* (Griseb.) Epling. *Revista Argentina de Microbiología* **33:**113–117.

Schnitzler P, Schon K & Reichling J (2001) Antiviral activity of Australian tea tree oil and eucalyptus oil against herpes simplex virus in cell culture. *Pharmazie* **56:**343–347.

Schuhmacher A, Reichling J & Schnitzler P (2003) Virucidal effect of peppermint oil on the enveloped viruses herpes simplex virus type1 and type 2 *in vitro*. *Phytomedicine* **10:**504–510.

Sinico C, De Logu A, Lai F, Valenti D, Manconi M, Loy G, Bonsignore L & Fadda AM (2005) Liposomal incorporation of *Artemisia arborescens* L essential oil and *in vitro* antiviral activity. *European Journal of Pharmaceutics & Biopharmaceutics* **59:**161–168.

Sivropoulou A, Nikolau C, Papanikolau E, Kokkini S, Lanaras T & Arsenakis M (1997) Antimicrobial, cytotoxic, and antiviral activities of *Salvia fruticosa* essential oil. *Journal of Agricultural & Food Chemistry* **45:**3197–3201.

Vijayan P, Raghu C, Ashok G, Dhanaraj SA & Suresh B (2004) Antiviral activity of medicinal plant of Nilgiris. *Indian Journal of Medicinal Research* **120:**24–29.

Vlietinck AJ, De Bruyne T, Apers S & Pieters LA (1998) Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. *Planta Medica* **64:**97–109.

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