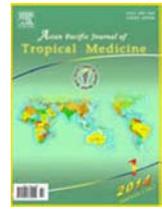




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Antiviral activity of maca (*Lepidium meyenii*) against human influenza virusJuana del Valle Mendoza^{1*}, Tomàs Pumarola², Libertad Alzamora Gonzales³, Luis J. del Valle^{1,4}¹Centro de Investigación de la Facultad de Ciencias de la Salud, Escuela de Medicina, Universidad Peruana de Ciencias Aplicadas–UPC, Lima, Peru²Servei de Microbiologia, Hospital Vall d'Hebron, Pg. Vall d'Hebron, 119–129, Barcelona 08035, Spain³Departamento Académico de Microbiología y Parasitología, Facultad de Ciencias Biológicas, Universidad Nacional Mayor de San Marcos (UNMSM), Avda, Venezuela s/n, Lima–1, Peru⁴Centre de Biotecnologia Molecular (CEBIM), Departament d'Enginyeria Química, ETSEIB, Universitat Politècnica de Catalunya (UPC) Barcelona Tech, Avda, Diagonal 647, Barcelona E–08028, Spain

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ABSTRACT

Objective: To investigate antiviral activity of maca to reduce viral load in Madin–Darby canine kidney (MDCK) cells infected with influenza type A and B viruses (Flu–A and Flu–B, respectively).**Methods:** Maca were extracted with methanol (1:2, v/v). The cell viability and toxicity of the extracts were evaluated on MDCK cells using method MTT assay. Antiviral activity of compounds against Flu–A and Flu–B viruses was assayed using a test for determining the inhibition of the cytopathic effect on cell culture and multiplex RT–PCR.**Results:** The methanol extract of maca showed low cytotoxicity and inhibited influenza–induced cytopathic effect significantly, while viral load was reduced via inhibition of viral growth in MDCK infected cells. Maca contains potent inhibitors of Flu–A and Flu–B with a selectivity index [cytotoxic concentration 50%/IC₅₀] of 157.4 and 110.5, respectively.**Conclusions:** *In vitro* assays demonstrated that maca has antiviral activity not only against Flu–A (like most antiviral agents) but also Flu–B viruses, providing remarkable therapeutic benefits.

1. Introduction

Influenza virus infection remains a major cause of morbidity and mortality during winter months almost every year. Influenza viruses can cause global epidemic diseases, known as pandemics, during which rates of illness and death from influenza–related complications can increase dramatically^[1,2]. High infection rates, short incubation periods, and rapid progression of the disease through the population are characteristic of influenza. Its hallmark is its epidemicity, and its impact on public health is dramatic. The final H1N1 statistics from Centers for Disease Control estimate that, between April 2009 and

April 10, 2010, there were between 43 million and 89 million cases of 2009 H1N1 of which 195 000 and 403 000 in 2009 H1N1 were related hospitalizations^[3]. The control of viral diseases has been a subject of intense scientific endeavor. There is an increasing need to find new molecules or compounds with antiviral activity for the treatment of viral infections because in recent years it has reported an increase of mutations to antiviral against Flu–A.

Influenza Type A (Flu–A) and influenza Type B (Flu–B) are two types of influenza viruses. Flu–A virus is further categorized into subtypes based on two surface antigens: hemagglutinin and neuraminidase. Vaccination remains the primary method for prevention of influenza, but vaccine strains must be continually updated and their protective efficacy is limited in people over 65 years of age who paradoxically are the group most severely affected by influenza^[4]. An alternative to failed vaccination is the administration of antiviral drugs. Two influenza inhibitors designed based on the structure of viral neuraminidase,

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zanamivir® and oseltamivir carboxylate®, have been approved for treatment and prophylaxis of influenza infection[5,6]. Amantadine® and rimantadine® are other chemically related agents with antiviral activity against Flu–A virus but not against Flu–B virus[7]. Amantadine has been used in the treatment and prevention of Flu–A virus infection for over three decades. Its use is associated with severity of symptoms and duration of illness[8]. Amantadine takes effects by blocking the ion channel activity of the matrix protein and inhibiting replication of Flu–A viruses. However, these viruses can rapidly become drug–resistant during treatment[9].

The aim of the present study is to evaluate *in vitro* the antiviral activity of *Lepidium meyenii* (*L. meyenii*) (maca) against respiratory viruses. *L. meyenii* (Walpers), also reported as *Lepidium peruvianum* (Chacón) and commonly known as maca, is a perennial herbaceous plant that grows in the Peruvian Central Andes (3700–4500 m of altitude). Maca has been consumed since the times of the pre–Incas and Incas. This tuber has an excellent nutritional value because of its high content in carbohydrates, proteins, vitamins and minerals[10]. Thus, maca is a powerful energizer, particularly indicated in cases of undernourishment or stress because it improves physical and mental energy[11]. Recently, diverse secondary metabolites of maca have been identified[12–14], and possible therapeutic applications to cancer[15], immune[16,17], and reproductive[18], disorders have been suggested. Also, for the first time ever, this work describes the antiviral activity of the methanol extract of *L. meyenii* against Flu–A and Flu–B viruses. Our results clearly show that this extract has a potent antiviral activity and would complement the use of maca as traditional medicine to treat respiratory virus infection.

2. Materials and methods

2.1. Plant material and extracts

L. meyenii (maca) was obtained in the Pampas Valley (Tayacaja, Huancavelica), in the Peruvian Andes. This plant is sold by neighboring farmers without the need of a marketing permit as maca is not considered a threatened or protected plant species. Maca root powder was macerated in pure methanol (1:2, v/v) for 10 d. The macerated mixture was filtered, and the eluted methanol was dried at 40 °C for complete evaporation of methanol[15]. Finally, dilutions were performed to evaluate antiviral activity. The same process was used to prepare an aqueous extract.

2.2. Cell lines

Madin–Darby canine kidney (MDCK) cells were obtained

from American Type Culture Collection, USA. The cells were grown in minimum essential medium with Earle’s salts (Gibco BRL, Grand Island, NY) supplemented with 10% foetal bovine serum, 25 µg/L gentamicin and 200 mmol/L L–glutamine (growth medium). Infected cells were maintained in minimum essential medium with 1% foetal bovine serum, 25 µg/L gentamicin and 200 mmol/L L–glutamine (maintenance medium). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂–95% air.

2.3. Viruses

Human influenza Type A virus (Flu–A or H1N1 strain A/New Caledonia/20/99) and Human influenza–B virus (Flu–B or strain Shanghai/361/2002) were obtained from the respiratory virus laboratory (Hospital Clínic of Barcelona, Barcelona, Spain) and used for *in vitro* assays as follows: the viruses were adapted and passaged more than twice in respective host cells (MDCK cells). When a cytopathic effect (CPE) characteristic of both viral infections was observed, the infected cultures were centrifuged at 3000 r/min for 10 min and supernatants stored at –80 °C until use. For virus titration, cells grown in 96–well plates were incubated at 37 °C for 2 h with decimal dilutions of virus suspensions. After absorption, the inoculum was removed and maintenance culture medium was added. Titres were calculated as 50% cell culture infectious doses (TCID₅₀)/mL to estimate endpoints by the Reed–Muench method[19]. All plates were incubated at 37 °C and observed daily to determine the CPE. Endpoints were estimated on the 6th day.

2.4. Cytotoxicity assay

Cytotoxicity of maca extract was assessed using an assay based on the color change subsequent to the reduction of 3–[4,5–dimethylthiazol–2–yl]–2–,–diphenyltetrazolium bromide (MTT) by mitochondrial enzymes[20–22]. The assays were performed using 1×10⁴ cell/well in 200 µL of medium which were cultured in 96–well plates and incubated at 37 °C in a humidified atmosphere with 5% CO₂–95% air. When cell cultures were confluent, the culture medium was removed from the wells, which were replenished with 0.2 mL of the maintenance medium containing maca extract prepared by dilution. Maca concentrations had a range from 0 to 1000 µg/mL. Each dose was assayed in quadruplicate. The wells with 0.2 mL maintenance medium but without virus samples were used as cell controls. All cultures were incubated at 37 °C for 6 d. Cell morphology was inspected daily for alterations. Cytotoxic concentration 50% (CC₅₀) is defined as the concentration of compound that reduces the viability of mock–infected cells by 50%. This index is commonly estimated by MTT assay. In our study, 20 µL of MTT stock solution (3 mg/mL in phosphate buffered saline) was added to each well. After 3 h of incubation under

culture conditions, the medium was carefully removed and formazan crystals were solubilized by adding 200 μ L dimethyl sulfoxide. Finally, cell viability was expressed as the percentage of the absorbance value determined for the control cultures. Absorbance (A570 nm) was measured in an ELISA reader. CC_{50} was then determined using Pharm/PCS software[23]. To confirm MTT results, the monolayers were also observed microscopically to estimate the CPE *i.e.* rounding and other marked morphological changes with respect to control cells[20].

2.5. Antiviral activity assay

Antiviral activity of compounds against Flu-A and Flu-B viruses was assayed using a test for determining the inhibition of CPE on cell culture[24–26]. MDCK cells were seeded at 10^4 cells/well in 96-well plates and allowed to attach overnight. The next day they were washed with phosphate buffered saline and inoculated with virus suspension at a multiplicity of infection of 0.01 except for the control well. Amantadine was used as positive control. After 120 min of incubation at 37 °C, 100 μ L/well of culture medium containing different concentrations of maca extract was added to confluent monolayers of cells. All cultures were carried out in quadruplicate and incubated at 37 °C in 5% CO₂ atmosphere for 6 d until control wells showed 70–100% CPE. All wells were then observed under a light microscope to determine CPE. Minimal inhibitory drug concentration that reduced CPE by 50% (IC_{50}) was calculated by MTT method[20,21].

2.6. Amplification of influenza virus by RT-PCR

A multiplex RT-PCR assay was performed to amplify highly conserved genes of Flu-A and Flu-B virus nucleoprotein[27]. Flu-A viruses were detected using primers: sense 5′-GAA CTC RTY CYW WAT SWC AAW GRR GAA AT-3′ and antisense 5′-ATK GCG CWY RAY AMW CTY ARR TCT TCA WAI GC-3′. Flu-B viruses were detected using primers: sense 5′-ACA GAG ATA AAG AAG AGC GTC TAC AA-3′ and antisense 5′-ATK GCG CWY RAY AMW CTY ARR TCT TCA WAI GC-3′.

Nucleic acids of both types of viruses propagated in cell cultures were extracted from 200 μ L of the sample by the guanidinium thiocyanate method[28]. After processing, the dry pellet was resuspended in 15 μ L of sterile RNase-free water.

RT-PCR amplification reaction was performed in a single step using the RT-PCR system kit (Promega), which consisted in a PCR mixture containing 2 mmol/L MgSO₄, 300 μ mol/L of each deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate and deoxythymidine triphosphate, 20 pmol of specific primers for Flu-A or Flu-B viruses, 10 μ L of 5 \times reaction buffer, 5 IU of cloned avian myeloblastosis virus reverse transcriptase, and

5 IU of Tfl DNA polymerase. An aliquot 5 μ L of RNA extract was added to a reaction final volume of 50 μ L. Amplification reactions were carried out in a PTC-200 (Peltier Thermal Cycler, MJ Research, Watertown, MA). Samples were subject to an initial cycle of 48 °C for 45 min, and then 94 °C for 3 min. The cycles conditions of the PCR were 45 cycles: 94 °C for 30 seconds; 55 °C for 1.5 min; 72 °C for 1 min, and a final incubation of 72 °C for 10 min. PCR products were analyzed by electrophoresis on 2% gel agarose (FMC, Rockland, ME) containing 3 μ g/mL of ethidium bromide in 1 \times Tris-borate buffer. The amplicon sizes were 721 and 991 bp for Flu-A and Flu-B, respectively.

3. Results

3.1. Toxicity of maca extract

The toxicity of the aqueous and methanol extracts of maca was determined using MDCK cells for virus propagation. MDCK cells were incubated with increasing amounts (from 0 to 1000 μ g/mL) of maca extract and cell viability was determined by MTT method. Relative viability of MDCK cells was calculated by comparison with untreated cultures (control, 0 μ g/mL). Results indicate that the aqueous extract did not produce adverse effects on MDCK cells (Figure 1). However, the methanol extract showed CC_{50} of 850 μ g/mL, but no toxic effect was observed in concentrations below 200 μ g/mL. These values were confirmed by microscopic observation of CPE. Thus, the methanol extract can contain the active compounds of maca to produce biological effects.

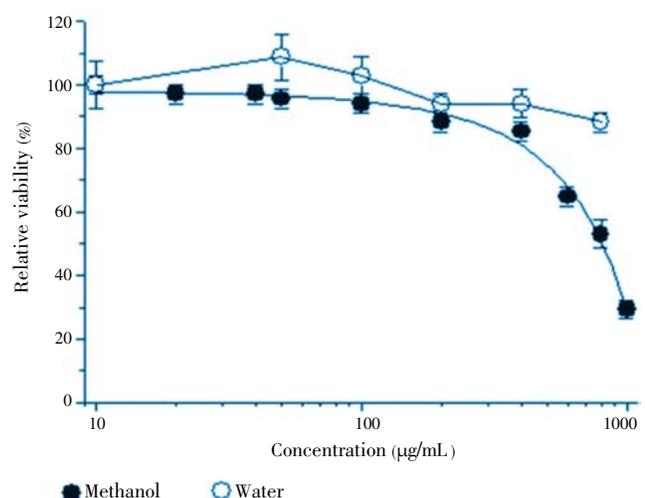


Figure 1. Cytotoxic effect of aqueous and methanol extracts of maca on MDCK cells.

Values are mean \pm SD of three independent experiments.

3.2. Antiviral activity of maca extract

The ability of maca extract to inhibit influenza virus replication in MDCK cells was examined. Cell viability often decreases during viral propagation. Thus, an increase in

Table 1Antiviral activity of methanol extract of maca against Flu-A (H₁N₁) and Flu-B on MDCK cells.

Compounds	Flu-A (H ₁ N ₁)			Flu-B			Reference
	IC ₅₀ (μg/mL) ^a	CC ₅₀ (μg/mL) ^b	SI ^c	IC ₅₀ (μg/mL) ^a	CC ₅₀ (μg/mL) ^b	SI ^c	
Maca extract	5.4	850.0	157.4	7.69	850	110.5	This study
7-O-galloyltricetiflavan	15.7	110.0	7.0	–	–	–	[29]
7,4-Di-O-galloyltricetiflavan	30.0	60.0	2.0	–	–	–	[29]
Quercetin-3-O-β-D-glucopyranosyl	24.5	392.5	16.0	–	–	–	[29]
Ribavirin ^d	62.5	>125.0	>2.0	–	–	–	[30]

a: IC₅₀ is the concentration of compound required to inhibit virus-induced CPE by 50%; b: CC₅₀ is the concentration of 50% cytotoxic effect; c: SI: CC₅₀/IC₅₀; d: Positive control drug.

cell viability means inhibition of viral propagation (antiviral effect). The methanol extract of maca exhibited a potent activity against Flu-A (Figure 2) and Flu-B (Figure 3) viruses. Concentrations in the range of 10–80 μg/mL were effective in producing an antiviral effect by re-establishing normal growth of MDCK cells. The half inhibitory concentration (IC₅₀) for Flu-A virus was 5.4 μg/mL (Table 1 and Figure 2a), a value found after determining cell viability of MDCK cells. Our results show a good correlation between IC₅₀ and the RT-PCR product of the viral nucleoprotein (Figure 2b). Commercial amantadine was used as positive control and had a clear inhibitory effect on Flu-A virus (Figure 2). It reduced viral load (Figure 2b), increasing cell viability of infected cells (Figure 2a). Flu-B virus replication was inhibited with an IC₅₀ of 7.69 μg/mL (Table 1 and Figure 3a). Viral inhibition was reflected by the decrease of viral nucleoprotein or viral load in MDCK cells (Figure 3b).

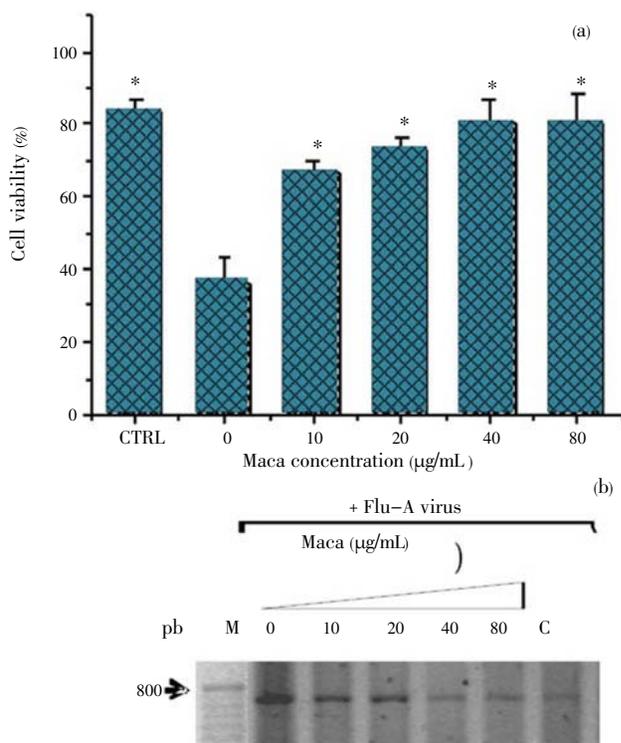


Figure 2. Antiviral activity of maca methanol extract against Flu-A. a: MDCK cells infected with Flu-A, then treated with methanol extract of maca. CTRL: treatment with amantadine (1 μg/mL); *: $P < 0.05$ compared with dose 0 using Tukey test; b: RT-PCR for viral nucleoprotein gene. C: cells treated with amantadine; M: DNA marker.

The selectivity index (SI), a therapeutic indicator, was calculated as the ratio between CC₅₀ and IC₅₀. SI values obtained for Flu-A and Flu-B are shown in Table 1. The SI determines the magnitude of the antiviral effect of the methanol extract of maca. Thus, this extract contains compounds with anti-influenza properties of therapeutic interest, especially against Flu-A.

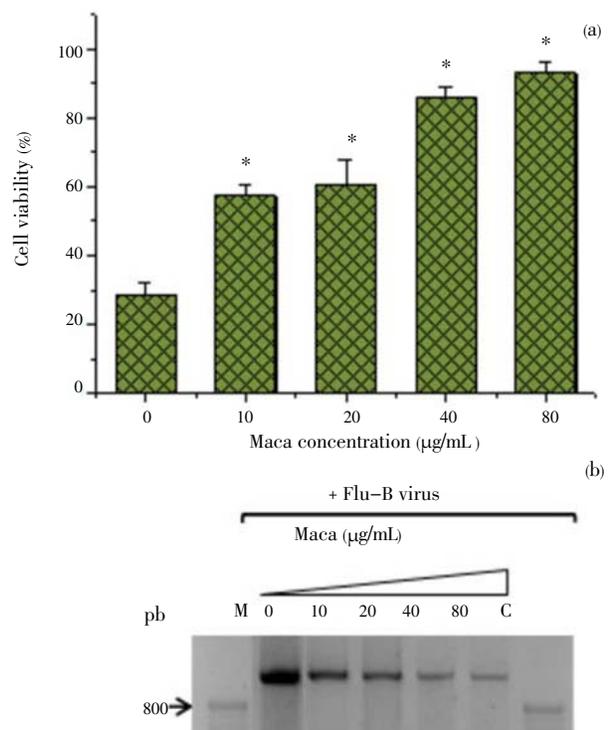


Figure 3. Antiviral activity of maca extract against Flu-B. a: MDCK cells infected with Flu-B, then treated with methanol extract of maca; *: $P < 0.05$ compared with dose 0 by using Tukey test; b: RT-PCR for nucleoprotein gene of Flu-B; M: DNA marker.

4. Discussion

Traditional herbal medicine has been used for the treatment of human infectious diseases in many countries for centuries. Combining with ethnopharmacology, it could be an alternative approach to the discovery of antiviral agents. The results of our study show that the methanol extract of maca has antiviral effects on influenza similar to those of amantadine antiviral.

Recently, two new antiviral flavan derivatives were isolated from leaves of *Pithecellobium clypearia* (Fabaceae). These compounds have antiviral activity against respiratory syncytial virus, Flu–A (H1N1), coxsackie B3 (Cox B3), and herpes simplex virus Type 1[29]. Other flavonoids obtained from *Avicennia marina* (Avicenniaceae) possess antiviral activity against HIV and herpes virus (HSV–1, HSV–2) inhibiting viral multiplication[30,31]. New flavonoids isolated from the antiviral fraction of *Wikstroemia indica* against respiratory syncytial virus[32]. Thus, it has been demonstrated that polyphenolic compounds may be effective against viral infections. In addition to these compounds, other constituents in plant extracts may contribute to antiviral activity[33], for example tannins, a group of secondary metabolites ubiquitous in plants[34].

Very few studies deal with antiviral compounds against virus. Tannins from persimmon (*Diospyros kaki*) extract have been reported to inhibit to 12 different viruses including both enveloped viruses (influenza virus H3N2, H5N3, herpes simplex virus–1, vesicular stomatitis virus, Sendai virus and Newcastle disease virus) and non–enveloped viruses (poliovirus, coxsackievirus, adenovirus, rotavirus, feline calicivirus and mouse norovirus)[35]. Our study clearly demonstrates that the methanol extract of maca can inhibit Flu–A and Flu–B virus growth and has a high therapeutic index compared to other antiviral compounds.

Now it is known that maca contains secondary metabolites with several medicinal properties, such as compounds for ovarian stimulation in rats to increase the number of ovarian follicles[15], and spermatogenesis stimulation in rats[18], and compounds with anti–oxidative properties[15,36]. These effects could be due to the presence of biologically active isothiocyanates (*e.g.* benzyl isothiocyanate and *p*–methoxy benzyl isothiocyanate)[29]. Other phytochemical components of maca with potential medicinal effects are essential fatty acids (*e.g.* linoleic and oleic), flavonoids and saponins[31], alkaloids[14], benzoyl derivatives[10], and glucosinolates[14].

Here we report that the methanol extract of *L. meyenii* (maca) has an inhibitory effect on Flu–A and Flu–B virus replication. Standard cell–based assays were conducted to determine this antiviral activity by quantitative analysis of flu virus replication, monitoring of CPE and detection of the conserved nucleoprotein sequence in flu viruses by RT–PCR, a very sensitive and rapid detection method for screening potential antiviral agents. Our results suggest that the antiviral activity of maca extract have several possible targets in flu viruses. The antiviral compounds of maca can interfere with virus attachment or prevent virus–cell fusion; therefore, the amount of envelop protein on the surface of infected cells is a key endpoint for infection control. Maca extract also decreases viral load

via post–entry of Flu infection.

In conclusion, since *L. meyenii* (maca) extract shows *in vitro* inhibitory properties against Flu–A and Flu–B viruses, it may represent a novel therapeutic treatment with antiviral activity for influenza infection. Finally, this study opens a new avenue for scientific inquiry on these effective compounds against human influenza.

Conflict of interest statement

We declare that we have no conflict of interest.

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