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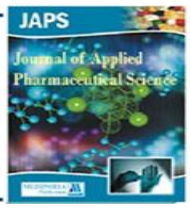

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Assessment of the cytotoxic effect and *in vitro* evaluation of the anti-enteroviral activities of plants rich in flavonoids

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ABSTRACT

Nanoparticles derived from plants known for their high content of flavonoids provide a biologically inspired route to designing therapeutic agents and a means of reducing nanoparticle toxicity. Little is currently known on the antiviral activity of *Salvia officinalis* L. and *Washingtonia filifera* H.Wendl aqueous extracts and their corresponding nanoparticles. In the present study, antiviral activity of subset extracts obtained from aerial parts of *Salvia officinalis* L. and *Washingtonia filifera* H.Wendl and nanoparticles derived from these plants were investigated against coxsackievirus B3 (CVB3). Different concentrations of extracts (7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL) were tested for their cytotoxic effect and the antiviral activity was carried out using three non cytotoxic concentrations against CVB3 in Vero cells. The cell viability was carried out using tetrazolium dye (MTT). The experiment was carried out by incubating the extracts with either virus or cells prior to infection. The silver nanoparticles derived from the tested plant extracts enhance antiviral activity at non cytotoxic concentrations. The biosynthesized nanoparticles may be further characterized to better evaluate their antiviral potential against other viral pathogens.

Keywords: Antiviral activity, Enterovirus, Nanoparticles, *Salvia officinalis*, *Washingtonia filifera*.

First and the second authors contributed eaually to this work.

INTRODUCTION

Flavonoids comprise a large group of plant secondary metabolites characterized by a diphenylpropane structure (C6-C3-C6). They are widely distributed throughout the plant kingdom and are commonly found in fruits, vegetables and certain beverages. Numerous preclinical and some clinical studies suggest that flavonoids have potential for the prevention and treatment of several diseases. One of the undisputed functions of flavonoids in plants is their protective role against microbial invasion. This involves their presence in plants as constitutive agents as well as their accumulation as phytoalexins in response to microbial attack (Harborne and Williams, 1992) Because of this protective role, it is not surprising that plants rich in flavonoids have been used for many years in traditional medicine to treat infectious diseases (Cushnie and Lamb, 2005).

The antioxidant properties of flavonoids are widely acknowledged (Pietta, 2000; Rice-Evans, 1996, 2001). They are known to have anti-inflammatory properties (Kim *et al.,* 2004). Flavonoid and its glycosides are examples of flavonoids that possess antimicrobial (McNally *et al.,* 2003; Baidez *et al.,* 2007), antibacterial (Basile *et al.,* 1999; Chung *et al.,* 2001; Lv *et al.,* 2009; Pettit *et al.,* 1996; Sato *et al.,* 2000; Sousa *et al.,* 2006; Tshikalange *et al.,* 2005; Xu and Lee, 2001; Yamamoto and Ogawa, 2002; Zhu *et al.,* 2004), antiviral (Tshikalange *et al.,* 2005; Yi *et al.,* 2004; Liu *et al.,* 2008; Wleklik *et al.,* 1988; Wu *et al.,* 2005; Li *et al.,* 2002) and antifungal (Zhu *et al.,* 2004, De Campos *et al.,* 2005 ; Sartori *et al.,* 2003) activities.

Salvia officinalis and *Washingtonia filifera* were characterized by their high content of flavonoids mainly luteolin 7-O-beta-D-glucoside, luteolin 7--O-beta-D-glucuronide, luteolin 3'-O-beta-D-glucuronide, 6-hydroxyluteolin 7-O-beta-D-glucoside, 6-hydroxyluteolin 7-O-glucuronide (Lu and Foo, 2000) and luteolin 7-O-glucoside 4''-sulfate (2), luteolin 7-O-glucoside 2''-sulfate (El Sayed *et al.,* 2006).

Myocarditis is defined as a subclinical inflammation of the heart muscle and may be induced by infectious, toxic or immunologic agents. Among the different infectious pathogens viruses are the most common causes of myocarditis (Kearney *et al*., 2001) and serological studies, nucleic-acid hybridization and PCR-based studies of endomyocardial biopsy and autopsy specimens have shown that enteroviruses represent one of the most common groups of viruses detected in the myocardium (Bowles *et al*., 2003). Although myocarditis is a frequent disease, it often goes unrecognized. Based on more than 12.500 randomly selected routine autopsies performed over a 10 year period about 1% of the population were found to have lymphocytic myocarditis (Friman *et al*., 1995). In most cases myocarditis is detected in the group of young adults between the ages of 20 and 39 (Rotbart and Webster). Coxsackieviruses belong to the family *Picornaviridae* and the genus enterovirus. The viruses have a positive-stranded RNA genome of about 7.4 kb encoding a monocistronic polyprotein (Kang *et al.,* 1994) which is processed into mature peptides during translation by viral proteases (Toyoda *et al.,* 1986). Four capsid proteins (VP1 to VP4) and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) are found during coxsackievirus infection of cells (Rueckert, 1996). Based on different organ tropism and differences in organ damage observed in mice, coxsackieviruses can be classified into two groups. The group A coxsackieviruses (CVA) has 23 members, whereas the group B coxsackieviruses (CVB) has only six members (Mahy, 2008; Melnick, 1996). Coxsackieviruses commonly induce mild disease, but under some circumstances, which seem to depend on genetic and individual predispositions, the viruses overcome local host defenses and can induce severe infections of the heart, pancreas, and brain (Melnick, 1996). The CVB group includes serotypes, in particular CVB3, that are frequently associated with infections of the heart. The course of CVB3 heart infections in patients may be different. Typically CVB3 induces acute myocardial infections and, in most cases, patients undergo a complete recovery. Fulminant infections, however, can occur and can result in sudden death. The aim of the present study was to evaluate the cytotoxic effect and the antiviral activity of *Salvia officinalis* and *Washingtonia filifera* collected from Tunisia and to carry out a comparative analysis of silver nanoparticles (NPs) derived from these plant extracts.

MATERIALS AND METHODS

Plant extracts and analysis

Washingtonia filifera fruits and leafs and *Salvia officinalis* were collected to make the aqueous extract. Extracts weighing 25g were thoroughly washed in distilled water, dried, cut into fine pieces and were crushed into 100 mL sterile distilled water and filtered through Whatman No.1 filter paper (pore size 25 µm). The filtrate was further filtered through 0.6 µm sized filters.

One mili-molar aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of NPs. Ten millileter of plant extract was added into 90 ml of aqueous solution of freshly prepared silver nitrate for reduction into Ag+ ions and kept at room temperature for 5 hours. The NPs were centrifuged at 6000 x g for 5 min. The pellet was washed twice with distilled water. The obtained NPs were subjected to UV-Vis spectra and granulometric analysis using Beckman-Coulter LS 230 laser granulometer in the 0.1-10000 nm range.

Cell culture and virus

The Vero cell line was maintained in RPMI 1640 supplemented with fetal bovine serum (10% v/v), L-Glutamin (2mM), penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. Coxsakievirus B3 Nancy strain (kindly provided by Pr. Bruno Pozzetto, Laboratory of Bacteriology-Virology, Saint-Etienne, France) was propagated in Vero cells.

Cytotoxicity assay

The evaluation is based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), by themitochondrial dehydrogenase of viable cells, to give a blue formazan product which can be measured spectrophotometrically. The MTT colorimetric assay was performed in 96-well plates. Cells were seeded in 96-well plates at a concentration of 5 x 10^4 cells/well and incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. Microscopic examination insured that stable normal cell layers were maintained in each well throughout every experiment. After treatment with various concentration of the test compound (7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL), the cells were incubated for an additional 48 h at 37 °C. The cells were examined daily under a phase-contrast microscope to determine the minimum concentration of compound that induced alterations in cell morphology. After that, the medium was removed and cells in each well were incubated with 100 µL of MTT solution (5 mg/mL) for 4 h at 37°C. MTT solution was then discarded and 50 µL dimethyl sulfoxide (DMSO) was added to dissolve insoluble formazan crystal and the plates were incubated at 37 °C for 30 min. Optical density (OD) was measured at 540 nm

using a Spectrophotometer reader (BIO-TEK® ELx800™ Universal Microplate Reader, USA). Data were obtained from triplicate wells. Cell viability was expressed with respect to the absorbance of the control wells (untreated cells), which were considered as 100% of absorbance. The percentage of cytotoxicity is calculated as [(A - B) / A] x 100, where A and B are the OD_{540} of untreated and of treated cells, respectively. The 50% cytotoxic concentration (CC_{50}) was defined as the compound's concentration (µg/mL) required for the reduction of cell viability by 50%, which were calculated by regression analysis.

Virus inhibition assay

Confluent Vero cell cultures were treated with Nanosilver and plant extracts during and after virus infection in two sets of experiments as follows: (1) 10^4 $TCID_{50}$ of the virus was exposed with effective minimal cytotoxic concentration of the samples for one hr at 37°C. Then 100 µL of the mixture was added to the cells cultured fluently in 96-well flat-bottom microtiter plate. Following one hr incubation at 37°C, the supernatants were removed and the cells were washed with PBS. Then 100 µL of medium was added to each well (Pre-penetration exposure); (2) 10^4 $TCID_{50}$ of the virus (100 µL/well) was added to each well. After one hr incubation at 37°C the unabsorbed viruses were removed and cells were washed and 100 µL of sample was added to each well (Post-penetration exposure).

All plates were incubated at CO_2-incubator for 48 hrs. The viability of the infected and non-infected cells was evaluated using absorbance values of formazan. The percent of protection was calculated as follows:

Percent protection = [(ODT) V - (ODC) V] / [(ODC) M - (ODC) V] ×100

Where (ODT) V, (ODC) V and (ODC) M indicate absorbance of the sample, the virus-infected control (no compound) and mock-infected control (no virus and no compound), respectively (Baidez *et al.,* 2007).

RESULTS AND DISCUSSION

Nanoparticle synthesis and analysis

The synthesized NPs, after the reduction of Ag+ by *Washingtonia filifera* leaf extract, showed a band with a pick at 1261 nm whereas NPs derived from *Washingtonia filifera* fruit extract showed three bands with three picks at 818 nm, 224 nm, and 67 nm. NPs derived from *Salvia officinalis* aqueous extract showed a broad band with two picks at 62 nm and 151 nm. This difference in size is due to the concentration of phenolic compounds which is critical to the size and morphology of metallic nanoparticles. Thus, the concentration of phenolic compounds in the *W. filifera* extracts might be the key determinant of the size of the resultant NPs.

Cytotoxicity and antiviral activity

The cytotoxicity of plant extracts on Vero cells was determined by calculation of CC_{50} which is 16.5 and 14 mg/mL for *Washingtonia filifera* H. Wendl. leaf and fruit extracts, respectively (Table 1). The CC_{50} was 22.7 mg/mL for *Salvia officinalis* L. leaf extract. The cytotoxicity of the biosynthesized NPs was increased for *Washingtonia filifera* H. Wendl. and *Salvia officinalis* L. leaf extracts with CC_{50} of 10 mg/mL and 11.7 mg/mL, respectively. The cytotoxicity of NPs synthesized from *Washingtonia filifera* H. Wendl. fruit extract was slightly decreased (14.9 mg/mL > 14 mg/mL). *Washingtonia filifera* H.Wendl. Leaf extract showed protective effect against virus infection that ≥ 25 % of cells did not show cytopathic effect at a concentration of 5 mg/mL. This extract was shown to be approximately twice more protective against virus infection when it was pre-incubated with virus prior to infection (IC_{50} = 5.8 mg/mL, SI = 2.8) than cell pre-treatment (IC_{50} = 15 mg/mL, SI = 1.1), where 44 % of cells remain viable. Silver NPs derived from *Washingtonia filifera* H.Wendl. Leaf extract were less effective against virus infection during cell pre-treatment (IC_{50} = 21 mg/mL, IC_{50} = 0.47). However, these nanoparticles exhibited higher protective effect against infection during virus pre-treatment (IC_{50} = 4.3 mg/mL, SI = 2.3).

The antiviral activity of *Washingtonia filifera* H.Wendl. fruit extract was shown in the case of cell pre-treatment prior to infection where at concentration of 5mg/mL, 55 % of cells were alive after infection. The pre-treatment of virus with plant extract did not shown any protective effect. Silver NPs derived from *Washingtonia filifera* H.Wendl. fruit extract significantly increased the antiviral activity (IC_{50} = 0.17 mg/mL, SI = 87.6), where 75 % of cells remained alive after infection when pre-treated with the NPs at a concentration of 5 mg/ml. In addition, an evident antiviral activity was shown when the virus was pre-treated with NPs (IC_{50} = 24.5 mg/mL, SI = 0.6).

The same findings were observed with *Salvia officinalis* L. leaf extract, where 53 % of cells remained alive after their pre-treatment with 5 mg/ml of plant extract (IC_{50} = 4.8 mg/mL, SI = 4.7). This protection was slightly decreased when the virus was pre-treated with the plant extract (IC_{50} = 5.9 mg/mL, SI = 3.8). The antiviral activity was increased using the NPs derived from Salvia officinalis L. whatever cells were pre-treated with the NPs (IC_{50} = 0.38 mg/mL, SI = 30.8) or the virus was pre-treated prior to infection (IC_{50} = 4.7 mg/mL, SI = 2.5).

The first essential step of viral infection is the interaction of the viral capsid with the receptor on the surface of the host cell. Coxsackievirus capsids are assembled from 60 identical protomers, each composed of the four structural proteins VP1–VP4. The viral shell is formed by VP1 to VP3, and VP4 lies on the inner surface and establishes a connection between the capsid and the RNA genome (Rueckert, 1996). The surface of the virion shows a five-fold axis of symmetry, surrounded by a large depression termed the "canyon" which contains a hydrophobic pocket (Muckelbauer *et al.,* 1995). The coxsackievirus and adenovirus receptor (CAR) is the primary receptor of CVB (Bergelson *et al.,* 1997) and its binding to the pocket on the canyon floor of CVB mediates the internalization of the virus into the host cell (He *et al.,* 2001). Antiviral compounds may selectively inhibit the interaction of CVB with CAR and thus prevent virus binding to the target cell receptors.

Table. 1: CC_{50}, IC_{50} and SI for *Washingtonia filifera* H.Wendl. and *Salvia officinalis* L.

Test samples	CC_{50} (mg/mL)	Concentration (mg/mL)	% Protection					
			Pretreatment of cells (%)	IC_{50} (mg/mL)	SI	Pre-treatment of cells (%)	IC_{50} (mg/mL)	SI
Washingtonia filifera H.Wendl. Leaf extract	16.5	5	25	15	1.1	44	5.8	2.8
		2.5	24			29		
		1.25	15			19		
Washingtonia filifera H.Wendl. fruit extract	14	5	55	4.9	2.85	0	ND	ND
		2.5	6			0		
		1.25	6			0		
Washingtonia filifera H.Wendl. Leaf NPs	10	5	10	21	0.47	55	4.3	2.3
		2.5	7			37		
		1.25	0			34		
Washingtonia filifera H.Wendl. Fruit NPs	14.9	5	75	0.17	87.6	27	24.5	0.6
		2.5	69			27		
		1.25	52			22		
Salvia officinalis L. leaf extract	22.7	5	53	4.8	4.7	41	5.9	3.8
		2.5	15			6		
		1.25	0			0		
Salvia officinalis L. leaf NPs	11.7	5	74	0.38	30.8	55	4.7	2.5
		2.5	60			17		
		1.25	55			6		

According to the obtained results in this study, the synthesized NPs have increased the antiviral activity of the tested extracts. The exception was for *Washingtonia filifera* H.Wendl. leaf extract which can be due to the potent cytotoxic effect of the plant NPs which was increased from 16.5 mg/ml to 10 mg/mL. The antiviral activity of this extract was carried out with a concentration of 5 mg/ml, so close to CC_{50}, and this value can be determined as CC_{25}, where 25 % of cells can be affected.

CONCLUSION

In conclusion, the antiviral activity of *Washingtonia filifera* H.Wendl. leaf extract and their corresponding NPs were more significant when incubating with virus prior to infection and therefore more effective against virus than cells whereas, *Washingtonia filifera* H.Wendl. fruit extract, *Salvia officinalis* L. leaf extract and their NPs were more effective against virus infection. However, it is not clear whether this activity is by acting on cell membrane and hampering virus interaction with cells or by preventing virus replication inside cells. The mechanism(s) underlying behind this activity remain to be understood.

As far as we know, the present work constitutes the first report studying the antiviral activity of *Washingtonia filifera* H. Wendl. and *Salvia officinalis* L. aqueous extracts and their corresponding NPs against coxakievirus B3. Further analysis need to be undertaken to isolate the bio-active molecules and the exact mechanism(s) underlying behind their activity. Plants and their chemical compounds are considered the main natural product used actually in pharmaceutical industries and therefore, it is very important to report the preliminary screening of their activities and safety to establish a good data base which may help for their better use. Researchers are looking the enhancement for the activity of plant essential oils and extracts using many approaches. The present study has discussed the possible use of $AgNO_3$ as a chemical agent which can enhance the activity of plant extracts.

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